

nature

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Rothschild's numerate arrogance

LORD ROTHSCILD, speaking on British television last week, argued that we should develop a table of risks so we could compare, say, the risk of our dying in an automobile accident with the risk of Baader-Meinhoff guerillas taking over the nuclear reactor next door. Then we would know how seriously to take our risks, be they nuclear power, damage to the environment or whatever. The 'econuts' (Lord Rothschild's usage) would soon be put in their place if we put numbers to the risks they envisage. What could be more reasonable than that? Has Rothschild lightened the darkness of the risk business with the light of science and number?

No. It is fine for Rothschild to demonstrate his agility with arithmetic, converting probabilities from one form to another (and implying that the viewers could not do it) but this is only the kindergarten of risk. He gave only the briefest of hints that there might be more to it when he called for errors to be quoted with risk estimates and when he remarked—in a brief aside—that occasionally one had to rely on expert assessment of risks, as in the case of nuclear reactors. But he threw these cautions away when he quoted not a single error on the risks he enumerated, nor developed the problem of the partisan 'expert'.

More than this, Rothschild confused two fundamental distinct kinds of risk in his table: known risks—such as car accidents—where the risk is simply calculated from past events; and unknown risks—such as the terrorists taking over a fast breeder—which are matters of estimating the future. The latter risks inevitably depend on theory. Whether the theory is a social theory of terrorism or a risk-tree analysis of fast breeder failure, it will be open to conjecture. And it ought to be remembered that the history of engineering is largely a history of unforeseen accidents. Risk estimates can be proved only by events. Thus it is easy for groups, consciously or unconsciously, to bend their calculations to suit their own objectives or prejudices. With unknown risks it is as important to take these into account as to come up with a number. Hence the danger of relying on 'experts' who are committed to a particular future. What is needed more than an expert table of risk values is a democracy of risk assessment, where the basic information on which conjectural risks

are calculated is made available to as many groups as possible.

Moreover some future risks are largely incalculable because we have no theory. What is the risk, for example, of a fast breeder economy leading to an erosion of civil liberties? This is a matter of legitimate concern, but how can Lord Rothschild's table deal with it? It is a conceit among some scientists that scientists alone are rational, and Lord Rothschild projected this conceit particularly clearly.

Peter Chapman, Director of the Open University Energy Group, caricatured this view when he spoke to the Parliamentary Liaison Group for Alternative Energy Strategies last week: "'rational' things, facts, are concerned with the reproducible aspects of the world. In the energy business this means the efficiency of machines, the heat loss from houses, the number of houses and so on. If we agree on the meaning of these terms we can agree how to measure them and hence have a basis for agreeing what to do. Anything that cannot be measured (and this included all my emotional reactions to things, people, and relationships) has to be left out. It is dubbed 'irrational' and excluded."

Science and numeracy are undoubtedly important parts of rationality, but they are by no means the whole of it; and scientists do a disservice to both by pretending that they are. In part they fail to distinguish the process of science from the product. Science in the making—like a projected risk—is always conjectural and hypothetical; it is confirmed by repeatability (part of the philosophy of a democracy of risk) and acceptance within the scientific community (a social event). It is never a matter of 'experts'; that is mere scholasticism. Future risks must be science in process for there can—by definition—be no experiment.

It is objective and rational to take account of imponderable factors. It is subjective, irrational, and dangerous not to take account of them. As that champion of rationality, the philosopher Bertrand Russell, would have argued, rationality involves the whole and balanced use of human faculty, not a rejection of that fraction of it that cannot be made numerical. By all means let us have numbers, where they can be agreed upon, but let us not be mesmerised by them. □



US to increase public participation in regulation of DNA research

SIGNIFICANT changes in procedures for regulating research involving recombinant DNA techniques are soon to be announced by the US National Institutes of Health (NIH). In particular, there will be greater public participation in both local biosafety committees and the NIH's Recombinant DNA Advisory Committee; and tighter provisions determining responsibility for ensuring that regulatory guidelines are observed.

Other changes expected to be announced are expansion of the scope of the present guidelines to cover recombinant DNA research carried out in institutions receiving support for such research from any federal agency (not just NIH); stricter rules about the public notice required to alter the guidelines; and increased provision for taking "risk assessment" considerations into account when deciding whether certain types of experiment should be exempt from the guidelines.

A revised version of the guidelines, which were first introduced by NIH in July 1976, were delivered to Secretary for Health, Mr Joseph Califano, at the end of last week. If he approves the changes, as is expected, then the new guidelines will be published in the *Federal Register* within a few weeks.

Proposed revisions to the guidelines were issued by Dr Donald Fredrickson, director of NIH, earlier this year. In particular, Dr Fredrickson has recommended lowering the levels of containment required for certain types of experiments (and to exempt other experiments from the guidelines completely) in the light of scientific evidence that has accumulated since concern over the possible social impact of recombinant DNA research was first raised five years ago.

Most of the suggested revisions to the scientific aspects of the guidelines are expected to be retained in the final version, apart from a few deletions from the list of exemptions. The major changes will occur in Part IV of the guidelines, that which covers "roles and responsibilities".

Much of the impetus for this change has come directly from Mr Califano, responding on the one hand to pressures from Congress—which is still threatening to introduce legislation to make up for any deficiencies it perceives in the guidelines—and on the other to public interest and environmental groups, which have been consistently critical of what they see as attempts to retain decision-making within the scientific community.

When NIH's proposed revisions were published, Mr Califano added an introduction specifically requesting comments on the proposed mechanisms for administering and revising the guidelines. Many such comments were aired at a public meeting held in Washington in September before a departmental review committee established by Mr Califano under the chairmanship of Mr Peter Libassi, chief counsel of the Department of Health, Education and Welfare.

According to sources in Washington, the major impact of the review committee's intervention and its study of the various comments and criticisms received, will involve aspects of public participation in the regulatory process, as well as channels of responsibility and accountability.

In his proposed revisions to the guidelines, for example, Dr Fredrickson had suggested that considerable authority for the conduct of recombinant DNA experiments be devolved from NIH to local biosafety committees; and that, in addition to including those with expertise in the relevant fields of science and public policy, at least one member of such a committee "shall not be affiliated to the institution."

Critics, such as Friends of the Earth and the Environmental Defense Fund, argued that this did not go far enough in involving the public in the regulatory process, in particular since the wording still allowed the external appointment to be made by the institution. The final version is expected to say that at least two members—or 20% of the total—should be appointed from outside, for example from environmental groups or local or state safety bodies.

Furthermore, membership of the Recombinant DNA Advisory Committee, another target of criticism, is to be increased from 14 to 20; the number of "public interest" representatives, is expected to be increased from two to six.

Additional changes to NIH's suggestions in the final version of the guidelines include clearly stated procedures for the director of NIH to adopt if it is proposed to make changes in the guidelines, specifying which changes need to be offered for public comment, which require advance notice, and which can merely be announced. Provision is also expected to be included for NIH to be able to remove the authority delegated to a local biosafety committee if it considers the committee's performance to be unsatisfactory.

One target of criticism in the suggested revisions was the proposal that, although certain types of experiments would continue to be prohibited, case-by-case exceptions would be allowed for experiments for which there were "compelling social or scientific reasons", provided that weight was given to "scientific and social benefits and to potential risks".

This language is now to be tightened up, so that the general standard for all such decisions will be an assessment that the experiment in question poses "no serious risk".

Two final points relate to the commercial aspects of recombinant DNA research. Firstly, the advisory committee is to be asked to consider what ground rules should apply to experiments involving more than 10 litres of culture containing altered genes.

All such experiments—not merely those involving "harmful products," as at present—would be prohibited, but still subject to waiver by the director of NIH; in commenting on this ban, the pharmaceutical company Eli Lilly told Dr Fredrickson that the necessity for such experiments was "imminent".

Secondly, reflecting a concern that a "voluntary" approach to regulating experiments in private industry suggested by NIH is likely to prove inadequate, the Food and Drug Administration is to consider whether similar guidelines should be applied directly to the pharmaceutical industry.

Many of the expected changes are likely to be welcomed by public interest and environmental groups, since they go some way towards meeting the criticisms of the guidelines on which such groups have been concentrating in recent months. "Any movement towards more responsible mechanisms for operating the guidelines is a step in the right direction," Dr Jonathan King, professor of biology at Massachusetts Institutes of Technology said last week.

Officials at NIH are said to be less happy, fearing that they are being pushed into a more regulatory role than many would wish, and are losing some of their flexibility to manoeuvre. Publication of the revised guidelines is thought to have been delayed by negotiations between NIH and DHEW.

Many of the changes answer criticisms given by Senator Adlai Stevenson, in a letter to Mr Califano last month, as reasons why he considered legislation still necessary. Mr Stevenson is said to be happier with the final version and the prospect of legislation may be receding.

David Dickson

US and Japan to cooperate in fusion and high energy physics

A BROAD programme for collaborative research in a number of areas of energy science has been agreed in principle by the United States and Japan. The programme, which will include fusion research and high energy physics, is expected to involve expenditure of about \$1,000 million by the two countries over the next ten years.

The main areas of collaboration were agreed during a visit to Japan in September by Dr James Schlesinger, Energy Secretary, and Dr John Deutch, head of the Department of Energy's office of energy research. The details were worked out in a series of meetings held in Washington last week; and it is hoped that an agreement will be signed within four months.

The two main items in the programme are research into magnetic fusion, and into liquefaction of coal. In addition, the two sides have agreed to cooperate in solar photovoltaics, geothermal energy, and high energy physics.

As far as fusion is concerned, the main focus of Japanese interest will be the General Atomic Company's large Doublet III programme in San Diego, to which the Japanese are expected to contribute about \$50 million.

In addition, the two sides have agreed to set up a joint research institute for fusion research. The Japanese end of the institute will probably be in Nagoya; no decision has been reached on a US site, with Massachusetts Institute of Technology and the University of California at Los Angeles being possible contenders.

In return for being allowed to participate in the fusion programme, the Japanese have agreed to contribute 25% of the costs of a \$700 million coal conversion demonstration plant planned for construction by Gulf Oil in West Virginia. The German government has already agreed to contribute a further 25%.

As for high energy physics, the two sides have in principle agreed on joint research and development into accelerator and particle detection technology and the joint construction and use of new facilities. It has been suggested for example that the Japanese may contribute to enhancing the 400×400 GeV intersecting storage accelerator (ISABELLE) at the Brookhaven Laboratory on Long Island. In the longer term, many US physicists are hoping that it may be possible to obtain joint funding for the next generation of particle accelerators. **David Dickson**

Home insulation may increase radiation hazard

SCIENTISTS at the University of California's Lawrence Berkeley Laboratories have suggested that the reduced ventilation in private houses resulting from conservation measures may pose a potential health hazard, by increasing exposure to low levels of the radioactive gas radon.

Radon-222 is produced as part of the decay chain of uranium-238. Both the gas and its short-lived decay "daughters"—in particular the alpha-emitters polonium 218 and 214—are present in the natural environment and are a recognised source of background radiation. Furthermore radon's precursor, radium-226, is a trace element contained in most rocks and soil; and indoor radon sources therefore include many building materials.

The health hazard is caused principally by radon's daughters, which can attach themselves to airborne particles and if inhaled, can lodge in the lung and emit short-range alpha radiation. In recent years, the hazardous aspects of high-level radon exposure have been dramatically highlighted by the high incidence of lung cancer among uranium miners. And only recently the federal government has been taking steps to minimise public exposure to the radon emitted by uranium mill tailings.

Six scientists at the Lawrence Berkeley Laboratory's energy and environment division have now suggested that, if medical data from the uranium miners is extrapolated backwards—and assuming a no-threshold, linear dose-response relationship at low levels of exposure—the decreased ventilation in private homes achieved in the interests of energy conservation could theoretically lead to an extra 20 to 200 annual deaths per million population from lung cancer.

New Spanish Constitution leaves science policy open

THE Spanish Parliament has just voted by a large majority (more than 90%) for the second democratic Constitution of this century. On 6 December the Spanish people are called to vote the text in a referendum. The main political forces, from extreme left to neofrancoists, will campaign for its approval.

Science is mentioned twice in the first part of the new Constitution. Article 20 recognises "the right to literary, artistic, scientific and technical production and creation" and article 44 says that "the public powers will promote science and technical and scientific re-

The scientists point out that despite recent interest in the effects of radon exposure in both Sweden and the UK, there is still little detailed knowledge about the effects of low level exposures over long periods of time, and that any conclusions are highly speculative. "We are not issuing a warning, but merely calling attention to a possibility that should be watched and studied", says Dr Craig Hollowell, one of the scientists concerned.

Despite this, a report produced by the scientists states that: "It is likely that some increased lung cancer risk would result from increased radon exposures; hence it is desirable not to allow radon concentrations to rise significantly".

In the long term, the scientists suggest that it may be necessary to include radon exposure levels in building standards. In the short term, they suggest a number of measures—such as sealing walls and floors, or coupling mechanical ventilation with heat exchangers—to reduce radon exposure.

The scientists have applied to the Department of Energy for further funds to carry out a more detailed investigation of the potential hazards—a request which has fallen between the department's environmental health and conservation responsibilities.

Some money has already been granted to study the radon emission of building materials. A complementary proposal for studying the health effects is being considered by the department's office of energy research. No one is yet prepared to admit that increased radon exposure does pose a health hazard, however small; but with growing concern about the long-term effects of low level radiation, no one is yet prepared to dismiss the hypothesis as unfounded. **David Dickson**

search for the benefit of general interest".

The mention of research in the articles dealing with the organisation of the State may be of greatest significance for scientists. One of the central constitutional problems has been the formation of autonomous governments in different parts of Spain. Article 148 lists the matters transferable to autonomous communities and it includes "the promotion of culture and research". However "the promotion and general coordination of technical and scientific research" can be found in the list of matters reserved

for central government. Nevertheless anything is possible because article 150 says that the central government may transfer or delegate to autonomous communities matters attributed to it.

The reason why the organisation of research is mentioned in the Constitution comes from the present unequal distribution of research centres in Spain. The question is left open because some people think that if control over research is given to autonomous governments research would be more

balanced and related to the needs of different areas; others fear that it could be more difficult for a smaller community to build an appropriate system of financing research and that co-ordination may not be easy.

Parliament will have to make a decision on these issues within the next few months. From a practical point of view it is very important that decisions are made quickly as the present provisional situation in science makes work very difficult. For instance

one of the main sources of research funds (FNDICT) may disappear next year and laboratories have not yet received the credits corresponding to applications made in 1977.

What remains to be seen is if political decisions will be taken soon enough to prevent the closing of certain laboratories and if the problems are treated with sufficient imagination to achieve an adequate structure for research.

Pedro Pulgomeché

Sweden's new government wrestles with nuclear power

NUCLEAR power has finally proved to be the undoing of Sweden's first non-socialist coalition government in 44 years. Former Prime Minister Torbjörn Fälldin handed his resignation to the Speaker on 5 October.

The government fell because some members of Fälldin's Centre Party opposed the coalition's decision on the loading of two reactors: Ringhals 3 and Forsmark 1. Under the Stipulation Law, the reactor owners could be given permission to load only after they had secured an acceptable reprocessing contract. They also had to show how and where the highly-radioactive waste resulting from reprocessing could be finally stored. Judging the application by these criteria, the government found "that the preconditions for consent are deficient in one respect. The application cannot therefore be approved".

The government went on to say that in order for the application to meet the conditions laid down for secure final storage, there should be test drilling to "show the existence of a sufficiently large rock formation of appropriate depth" with the qualities already prescribed by the nuclear industry's Nuclear Fuel Safety (KBS) project*. If the applicants found rock they considered suitable and applied again, the government would ask the Nuclear Power Inspectorate to judge the application. If the Inspectorate approved it, the government would give permission for the reactors to be loaded.

This agreement was immediately interpreted by the Press as a 'soft yes' to the loading, as it was assumed that the test drilling was a mere formality which would quickly produce the required rock. The Centre Party was criticised for yet another capitulation: although anti-nuclear, it had made several compromises on the issue during the government's term. How much influence these attacks had is uncertain, but, at the insistence of former Energy Minister Olof Johansson and other members of the Centre Party, Fälldin demanded that his coalition partners should agree to a referendum

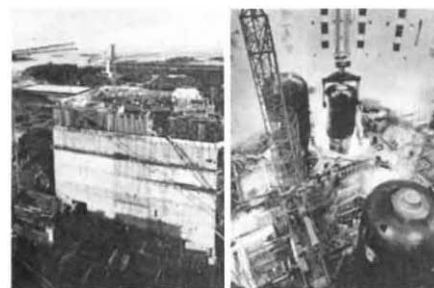
on the issue. They refused, and the government fell.

After much speculation, a minority Liberal party government was sworn in on 18 October. The new Energy Minister, Carl Tham, favours nuclear energy, but his statements since becoming Minister have been cautious.

Tham has said that the investments already made in nuclear reactors should not be wasted, and that those already in operation or being constructed should be used if security demands can be met. This would mean drawing the nuclear line at eleven reactors. He also wants to tighten up security on all energy forms. The new government will, he says, follow the old one's agreement on the Ringhals 3 and Forsmark 1 reactors.

Since the government's fall, debate has raged over the extra drilling requirements. Will they prove to be a simple formality, or was this a subtle way of delaying the two reactors perhaps for years? Engineer Lars Bertil Nilsson, chief of the KBS project, is confident that suitable rock will be found quickly. "There are certainly many places in Sweden where the rock is suitable", he says. "According to the government's statement, we simply have to find one of them. We don't need to choose any particular place now for final storage: that's a long-term business and will need to take a lot of other things into account besides the rock itself: buildings, transport, public opinion, etc. All we have to do now is to show that suitable rock exists. It is our judgement that we need only drill four new holes at Finnsjön, near Forsmark, and three new ones at Karlshamn. The drilling has just begun and will be finished by the end of the year. Then we'll put our report together and make a new application to the government in 1979."

His optimism is not shared by various researchers and some of the geologists actually doing the drilling. They have claimed that reliable results cannot be expected before two to ten years.



Forsmark 1 and Ringhals 3 under construction.

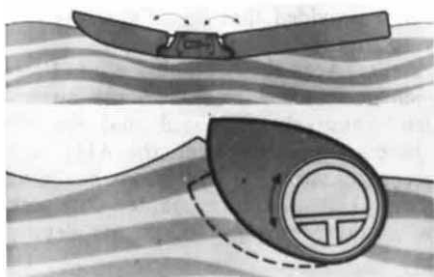
Meanwhile, the Nuclear Power Inspectorate is preparing itself for the new application. According to Dr Thomas Johansson, Vice-Chairman of the Inspectorate's Board, an expert panel of geologists is being set up to review the application. Before it is presented, however, the board is asking the panel to discuss guidelines for its evaluation. The panel should list the qualities that the nuclear power industry has said suitable rock should have; discuss possible measuring techniques that could be used to claim that the rock actually has the required characteristics; and decide what constitutes "showing" that the rock is suitable. "None of the geologists on the panel is involved in the drilling", says Dr Johansson. "The intention is to have a completely independent review."

What, then, of the future? "I am rather pessimistic", says anti-nuclear activist Björn Gillberg. "The Centre Party committed suicide when it passed the Stipulation Law. Whether or not we want nuclear energy should be decided on very broad grounds: social, political, psychological, environmental, international. To reduce it to the technical handling of waste, as the Law does, simply turned out to be a trap. The Centre Party didn't realise it had set a trap for itself until it was too late. They're nice guys, but they're naive. I can see the [pro-nuclear] Social Democrats winning next year's elections."

Wendy Barnaby

**The first KBS report, against which the Ringhals 3 application was judged, is called "Handling of Spent Nuclear Fuel and Final Storage of Vitrified High Level Reprocessing Waste". It is available from KBS, Brahegatan, S-102 40 Stockholm.*

Enthusiasm for UK wave power survives high costs



The Cockerell raft (top) and Salter duck

WAVE energy is not the white knight rescuing the fair society. That seems certain. Nevertheless the participants at a conference on the UK wave energy programme in London last week were optimistic about their ability to solve eventually the formidable problems of harnessing the power of the waves through steady work. And they were equally optimistic about the value of doing so.

The bad news about wave energy is that estimated costs per kW installed for the UK are £5,000-£9,000 compared to £500-£1,000 for a fossil or nuclear station. Current devices are a factor of 20 too costly for the value of the energy they can produce, concludes a report prepared by the consulting firm of Rendell, Palmer and Tritton in conjunction with Kennedy and Donkin. Referred to as a 'hatchet job' by observers outside the wave energy programme, the RPT report, commissioned by the Wave Energy Steering Committee, does not appear to have lowered morale. As Dr Stephen Salter of Edinburgh University, designer of one of the principal devices, the Salter duck put it: "Sir Hermann Bondi (Chief Scientist in the Department of Energy) has given us the best wings money can buy. RPT has told us how hard the ground is and the rest is up to us." Clive Grove Palmer, Programme Director of Harwell's Wave Energy Group told the conference: "This group thrives on problems and challenge. They won't just lick them, they'll eat them."

The high cost of the early designs arises from the diffuse character of the waves themselves. To capture the energy, large areas of device have to face the on-coming waves. With either concrete or steel the designs have been expensive. "But remember", says Grove-Palmer, "the Wright brothers' first estimate of the cost of air travel was \$30,000 per mile and they went right ahead".

The UK is favoured with some of the best off-shore sites in the world for the exploitation of wave power. Energy in waves is governed by wind strength

and length of water over which the wind blows. Prevailing Westerly winds blowing across as much as 1,000 miles of the North Atlantic create waves of average power of 50 kW per metre of wave front.

Since the UK wave power programme is only two years old and since the conference participants have an obvious enthusiasm for what has been accomplished with the first generation devices, Grove-Palmer's optimism may be justified. What, then, has been achieved in two years?

Two of the main devices are the Cockerell raft and the Salter duck. The Cockerell raft uses the relative angular motion of two segments of raft and the Salter duck uses a similar action of oscillating pods about a common spine. The precise mode of conversion is a subject of study. The rocking action can be used to drive a pump which can pressurise a fluid which can drive a turbine. But other proposals include on site conversion to hydrogen, on site desalination or direct use of the mechanical power to make heat.

Other devices under active consideration are the oscillating water column (OWC) and the Lancaster University flexible bag. The OWC is a box with a hole in the top. The waves make the water level oscillate and air is forced through the hole to drive a turbine. The flexible bag is potentially the cheapest design. Air filled bags are attached to a submerged hull. The wave crests collapse the bags and the displaced air drives a turbine. The bags are filled during wave troughs from a low pressure duct.

Grove-Palmer detailed six areas of future work in his closing summary.

- The need for better wave data. The irregularity of the waves in height and direction needs study. According to M. J. Platts of Wavepower Ltd, the builders of the Cockerell raft, one difficulty is that the oil industry has kept its accumulated wave data from the North Sea platforms 'inaccessible'. Platts has been unable even to arrange a visit to one of the platforms.
- Detailed critical engineering studies of how best to extract the power.
- Spine articulation of Salter's ducks. The bending moments are severe.
- Tests of the oscillating water column in real sea conditions.
- Further tests of the flexible bag because of its low cost.
- Mooring. Existing mooring equipment has been designed for ships and is unsuitable. Equipment is needed to withstand powerful mooring forces in high seas, corrosion and fouling by marine organisms over a period of 25

years without inspection. (Ships get drydocked every three or four years).

● Size and survival in high seas.

Aside from the technical problems are the problems of actually producing a large scale operational system. M. J. Platts estimates that all the design problems need to be solved and a fully operational production system needs to be built before 1990 if there is to be significant (20%) wave power production by 2020. After 25 years, production must be entirely given over to replacement so building up sufficient capacity before that time is a problem. Platts also points out that on this time scale one can expect significant social and economic changes which are impossible to predict.

A novel feature of the wave energy programme is the attention now being paid to environmental and social aspects. Preliminary estimates indicate that the effect of wave energy devices 10 km off shore will be to increase the size of beaches and the economic well being of the local population. But the lobster, herring and the £20,000,000 salmon industry are concerned that installation of the devices could interfere with the normal migration and mating behaviour of the fish. A third nuisance could be the possibility of seals finding the devices habitable with unknown consequences.

Problems of interfering with shipping are more serious. There are problems of collisions, the generation of rougher seas by wave reflections from the devices, and the need for reliable warning lights and radar reflectors to mark gaps up to 2 km wide to a standard much higher than ever attempted in the open sea.

To many wave energy is an attractive alternative energy possibility. "There is still a major amount of work ahead before conclusions can be reached about the future of the technology" said Dr F. J. P. Clarke, Chairman of the Wave Energy Steering Committee. But realism must tell us that any talk of a full scale or near full scale wave energy station out at sea by the early 1980s is premature. We still have to evolve concepts and engineering designs that would justify the major expenditure involved in such a step." Clarke tempers his judgement with a more positive note. "The resultant [wave energy] team... comprises a substantial national asset, and given continuous government support it is determined to find solutions. The natural resource is there and if anyone can learn how to capture it this team will."

Joe Schwartz

news in brief

Pochin calls for improvements in safety at Aldermaston: Substantial improvements in safety measures are urgently needed at the Atomic Weapons Research Establishment, Aldermaston, according to Sir Edward Pochin in his recent report on safety at AWRE. Workers there should be better protected from inhaling radioactive dust, the report says.

Pochin's inquiry, leading to the recent report, was set up following events last August when three women from the special laundry at AWRE were found to have plutonium in their lungs in excess of the level recommended by the International Commission on Radiological Protection.

In the report, Sir Edward says that the general level of health protection in the plutonium processing buildings is "of borderline adequacy", even though the industrial safety record is excellent.

Cutbacks in the number of safety staff and inadequate training had caused a serious decline in monitoring of radioactivity, he says, and, consequently, workers were being exposed to potentially dangerous levels of radioactivity. Monitoring standards had fallen behind those in other establishments in the UK and the US, although an increase in the number of personal air samplers, and a body counter to estimate plutonium in the lung, now being built, would improve matters.

Sir Edward recommends that in the long term major improvements should be made to buildings where solid and liquid waste are reprocessed; the containment of alpha-emitting radionuclides is very difficult with the present safety measures, he says.

AWRE and the National Radiological Protection Board have set up a joint committee to review the radiation exposures of individual workers, and inspectors from the Health and Safety Executive are to visit the establishment.

Supreme court rejects Podrabinek's appeal: Aleksandr Podrabinek (pictured right), the Moscow paramedic sentenced last August to five years internal exile last week had his appeal rejected by the Supreme Court of the Russian SFSR. The court stated that the charges of anti-Soviet slander had been upheld.

Mr Podrabinek, an active member of the illicit "Working Commission for the Investigation of the Use of Psychiatry for Political Purposes" is the author of a 265-page *samizdat* dossier on Soviet abuses of psychiatry to crush dissent.

Mr Podrabinek was not permitted to attend the appeal hearing. Nor was his chosen defence counsel, the London lawyer Louis Blom-Cooper. According to Mr Blom-Cooper Podrabinek's original trial involved the violation of no less than 78 provisions of Soviet legal procedure. Dissident sources in Moscow suggest the same pattern was followed at the appeal hearing—no defence witnesses were called nor was Podrabinek's own statement read to the presiding judges. (Photo courtesy of Keston College.)



belongs to the researcher provided that he or she was not employed specifically to invent.

After the 1977 Patents Act, the Committee of Vice-Chancellors and Principals issued a report on the ownership of patents which tentatively suggested that the individual should hold patent rights. However, the AUT says that in the past most compensation for inventions has gone to the universities. The AUT, therefore, wanted "to seek an independent view", said Mr John Akker, its deputy general secretary.

The AUT hopes soon to begin talks with the Committee of Vice-Chancellors and Principals to lay down guidelines on the ownership of patent rights which could be applied to all British universities.

NASA is to investigate Martian "White Rock"

This strange, white, horseshoe crab-shaped feature found inside a Martian crater (see photo) is to be studied by scientists working with the NASA Viking mission. The feature, nicknamed 'white rock', is the only one of its kind on Mars. Its composition is unknown, but it is unlikely to be either ice or snow because it lies near the equator.

The 'white rock' is about 18 km long and 14 km wide, and is at the bottom of a crater located at 8° South, 335° West.



Women scientists still lag in US: Despite significant increases in the proportion of women science graduates emerging from US colleges and universities, their salaries are still lower than those of men with comparable training and experience at every age, every degree level, in every field and with every type of employer (except for chemistry BAs employed by industry), according to figures released last week by the US Scientific Manpower Commission.

The commission says that detailed statistical analysis of the participation of women in the professional labour force indicate that women have roughly doubled their proportion of earned degrees in the sciences since 1970, and quadrupled their share of engineering bachelor's degrees. However, unemployment rates for professionally trained women continue to be two to five times higher than for men in the same field with the same level of training, and the gap increases at higher levels.

In the federal government, women still lag well behind their male counterparts in grade level (and hence salary). Thus among the federal government's 1,700 microbiologists, the average salary is \$23,260 for men and \$18,550 for women.

New parliamentary group for alternative energy: The UK Parliamentary Liaison Group for Alternative Energy Strategies called its first public meeting last week at the House of Commons. Chaired by Frank Hooley, MP, and supported by many environmental groups, the Group called Sam Berman, Director of the Lawrence Berkeley Laboratories, Peter Chapman, Director of the Open University Energy Group, and Gerald Leach, of the International Institute for Environment and Development, to speak. The next meeting is on 29 January with Tony Benn, Secretary of State for Energy.

Patent rights for university researchers: In an attempt to clear up the issue of whether patent rights for inventions made by researchers in British universities should be held by the researcher or the university, the Association of University Teachers (AUT) has sought the opinion of a leading firm of solicitors, which has said that the invention

Health and safety research gets underway

FEW research institutes would complain about a yearly budget increase of more than 40%; fewer still would be able to approach this level. But last year the HSE spent £8.5m on 'research' testing, and scientific support activities, compared to £6m in the previous year. There is, however, good reason for the executive's windfall, as the 1977 research report makes clear. The HSE is a relatively new institution, brought into being by the 1974 Health and Safety at Work Act, and so is still growing.

The Act established a Health and Safety Commission (HSC), a body with the task of determining policy for health and safety in Britain. A tripartite organisation consisting of trade union, employer and local government nominees, the commission has a role which can be compared with that of a company board of directors, with the basic difference that of all its members, only the chairman is a full-time employee. The HSE was formed to implement the commission's decisions, and is responsible for the day-to-day policing the HSW Act.

All people at work—with the exception of domestic workers in private employment—are covered by the Act. The HSE required facilities from several of the more established ministries in order to carry out its obligations. The Alkali and Clean Air Inspectorate was transferred from the Department of the Environment, a move vigorously opposed by the DoE. Other transfers, from the Departments of Energy, Employment, and, Prices and Consumer Protection and the Ministry of Agriculture, Fisheries, and Food, were achieved more easily.

It is hardly surprising, therefore, that the HSE was much involved with its own organisation during 1975 and 1976. The report indicates that

The UK Health and Safety Executive (HSE) last week published its research report for 1977—the most comprehensive yet to be published on the executive's research activities. **Alastair Hay** here looks at the research labs run by the HSE.

1977 was the first year that the HSE could concentrate its efforts on new areas of research, a situation reflected in its budget. It is also pointed out that some 28% of the £8.5m was allocated to extramural work, with the aim of providing the executive with additional expertise and facilities not immediately available to it.

These are the Health and Safety Laboratories (HSL) run by the HSE. The Explosion and Flame Laboratory (EFL) is based at Buxton; the Occupational Medicine and Hygiene Laboratory at Cricklewood; and the Safety Engineering Laboratory in Sheffield.

Explosions and flames

Dr Herbert Eisner is the Explosion and Flame Laboratory's director. Outlining the work carried out at Buxton, he indicated that much of the research is concerned with the development of test methods, pointing out that "If a hazard is present, you need to discover the mechanism which caused it".

Of particular concern to EFL are hazards encountered in gases. The reason behind the EFL's interest in gas is obvious from its history. It started life over sixty years ago as the Safety in Mines Research Establishment and in an even more remote location, in Cumberland. From its in-

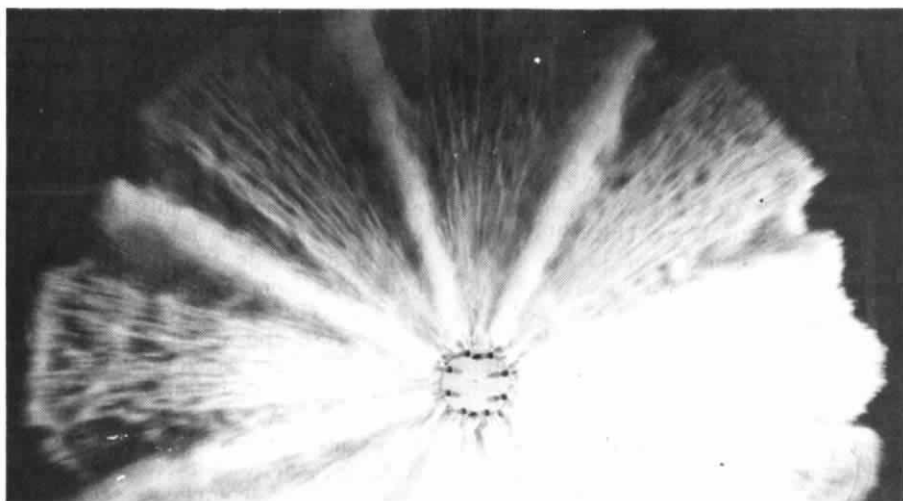
ception, the laboratory has been concerned with the prevention of explosions in coal mines and the development of safe electrical equipment for use in explosive atmospheres. Research of this nature has a far wider application than that of coal mining alone: spark-proof equipment is vital in many industries. Eisner selected two at random: fork-lift trucks used in the chemical industry, and safe diesel engines which can be run in potentially dangerous atmospheres.

Many government agencies are moving over to the system by which they choose at cost. The EFL does make charges at a commercial rate, although—as Eisner points out—the information provided is cheap at the price. Invaluable work has been done in the area of the testing of explosive devices for use in industry. Manufacturers of explosives have a difficult task: they are required to develop explosives which are safe for use underground, and which in many cases release an energy 10⁸ times greater than that required for the ignition of any methane which may be present.

Some of the current research programmes at the EFL include computer model studies of uncontrolled vapour clouds and explosions and, following the recent grain silo accidents in the US, the explosive potential of these installations is receiving more urgent attention.

Although there has been no explosion involving coal dust in a British mine for the past fifteen years, there is no ground for complacency, according to Dr Donald Rae. Rae is one of the physicists at Buxton and is investigating automatic devices for the containment of explosions underground, and he maintains that there are occasional situations in which the stone-dust barriers in current use need to be replaced. The high rate of coal extraction in modern mining apparently makes automatic devices essential. Stone-dust barriers require a great deal of maintenance; Rae feels that the 'triggered barrier' could be the answer for the future. He and his colleagues have spent ten years developing this device, and the first will soon be installed in a British mine. At present stone-dust is tipped into the path of an explosion to 'snuff it out'. The triggered barrier uses water under high pressure to perform the same function. A thermocouple responds to the flame of the explosion, relaying the message to a pressurised nitrogen cylinder, the pressure of which is used to eject water from a second cylinder into the path of the oncoming flame.

The possible range of flame speeds



Discharge of water from disperser of triggered barrier.

underground is anything between 0.4 and 3,000 m s⁻¹ (detonation speed). In practice, most explosions in their early stages travel at a speed of between 40 and 400 m s⁻¹, and the barrier has been developed accordingly and will ensure that sufficient water is available to extinguish anything in this range.

The NCB was swift to realise the ignition hazard posed by light metals, although mining industries in some countries have still not taken action to reduce, or indeed eliminate, the hazard—some mines in the US are known to fall into this category. Frank Powell and Roy Gregory, both working at Buxton, demonstrated all too easily how a 'spark proof' tool with a handle of aluminium, or other light metal, will produce showers of sparks if struck on a rusty steel bar. With their high affinity for oxygen, these metals, when so struck, cause a thermite reaction, generating temperatures of about 3000 °C; the sparks which are simply the visible hot fragments of reacting metals may have sufficient energy to ignite methane, and would present an ignition risk if the metals were allowed into a mine. It is with some justification therefore, that Powell and Gregory tend to be rather dismissive about the safety qualities of some 'spark proof' tools. And they add that it is not only the mining industry which has been shown to be at risk: for light metal anti-corrosion electrodes have caused several explosions in oil tankers.

Safety engineering

The Inquiry into the explosion at the Flixborough Chemical plant in 1974 concluded that poorly constructed and insufficiently supported by-pass piping at the 'Nypro' plant collapsed, allowing cyclohexane vapour to escape. Ignition of the vapour produced an explosion which was responsible for the death of 28 people. The Safety Engineering Laboratory (SEL) at Sheffield played its part in the investigation. The work at Sheffield included forensic studies of the failed components and materials, involved as well as tests on a simulated by-pass pipe assembly.

Entering the Incidents Laboratory at SEL is rather like visiting the yard of a scrap metal dealer: lifting gear, process-plant components, mining equipment and wire ropes are strewn over the floor of the lab. Jack Deakin, an engineer in the laboratory, says that most of the items were removed from the site of accidents and either had been, or were about to be, examined by the laboratory. The investigations done cover fatigue and brittle fractures, defective welding, and design and maintenance problems.

But engineering is not the only concern of the Sheffield Laboratory. For

the past few years fatal accidents in the mining industry have remained at about 50 per annum, following a steady fall over the previous 20 years as conditions in the mines improved. Indeed, the fatal accident rate 100,000 man-shifts worked was almost halved between 1958 and 1971, but since then there has been no significant improvement. And the fall in fatalities has not been matched by a decline in the serious injury rate.

Dr Clive Nussey (of SEL) believes that studies of minor accidents and 'near miss' situations might well provide information to help reduce the fatal and serious injury rate. (The chemical industry has recognised for a long time that the lessons to be learned from 'near misses' are as valuable as actual accidents, particularly as the former occur so much more frequently than the latter). Nussey says that over a third of the fatal and serious injuries in the mining industry are attributable to haulage and transport. And in a collaborative study on minor injuries associated with transport and handling accidents, done with the NCB and HSE's Mines Inspectorate, Nussey's laboratory found that the events leading to these injuries, or indeed to 'near misses', also appear as causes of fatal or serious injuries on transport systems. Nussey believes, therefore, that even outside the mines the frequency of these events is such that they could form the basis of early warning system to help predict where more serious injuries might occur in future.

Medicine and hygiene

The Occupational Medicine and Hygiene Laboratory is the third of the HSL Laboratories and is mainly located at Cricklewood. The biological monitoring work in this laboratory is under the direction of Dr David Gompertz; he is relatively new to the post, and is still in the process of developing the laboratory facilities. He admits to being "aggressive and demanding" and to being impatient to change the emphasis of the work in his laboratory. When Gompertz moved in, 85% of the work of the Occupational Medicine Laboratory was on metals and only 15% on organic chemicals; and 95% of the time was spent doing routine investigations. This will change.

It is envisioned that in the future only 40% of the work will deal with metals, and 60% will be concerned with organic chemicals—but this does not mean there will be less work on metals. The routine investigations will still consume most of the time—about 60%—but Gompertz says that the rest will be devoted to research and development. Gompertz is receiving support from the HSE for his programme of change.

Evidence of that support is provided by one of the laboratories latest acquisitions, a gas chromatograph and mass spectrometer which will be used to build up a background library of chemicals for use in the biological monitoring of workers at risk.

The Occupational Hygiene Laboratory (OHL), also at Cricklewood, is much concerned with the measurement of contaminants—such as fibrogenic materials, toxic metals, gases and vapours—in the working environment. Work at the OHL centres on developing the methodology and instruments for use in such situations. One of the instruments developed there, a general-purpose tape for monitoring atmospheric chemicals, is listed in the HSE's 1977 Research Report. The few tapes currently commercially available are designed to have a long shelf life and to remain stable. In consequence, they can only be used for a limited range of chemicals. The HSE research team has now developed a more versatile tape with a wider application. Although the report devotes no more space to this development than it does to other topics, this in no way lessens the impact for occupational hygienists. The institute is also about to publish details of a tape for detecting aromatic amines; and hopes to produce ones for aromatic isocyanates, phosgene and chlorine.

The technology for measuring respirable dust concentrations is constantly being improved and the OHL is placing much emphasis on another of its entries in the report, a continuously recording instrument for measuring dust samples. Called SIMSLIN II—the Safety in Mines Scattered Light Instrument—it is an improved version of an earlier model. SIMSLIN II is currently at the pre-production stage and is being tested in underground trials in co-operation with the NCB. SIMSLIN II weighs 8.2 kg and is 42 cm long and according to the report it is 'intrinsically safe in methane atmosphere'. The instrument is able to exclude non-respirable particles from the dust intake and to measure the respirable fraction by light scattering at regular—15 s or longer—intervals. With SIMSLIN II already being manufactured by a commercial company the OHL must be fairly certain of its marketability for it asserts in the report that "the instrument should find wide application in many industries where detailed records of dust concentrations are required".

The HSE appears to be acutely conscious of the need to produce useful research findings in the field of health and safety. As it is, the 1977 report forms an impressive track record for a research year, and there are unlikely to be many complaints about the chosen allocation of the HSE budget. □

HSE's scientific mediator

On 1 July this year, Dr Ken Duncan became the third member of the triumvirate that runs the Health and Safety Executive (HSE). Duncan will be the principal person at HSE concerned with matters of biomedical research. He will combine his new work with his job of the past four years as Director of Medical Services on the staff of the HSE secretariat. Here he talks to ALASTAIR HAY.

OCCUPATIONAL medicine has been Ken Duncan's main concern for much of his working life, most of which has been spent in industry. His career began with British Rail and the South Western Gas Board. He later became chief medical officer of the United Kingdom Atomic Energy Authority and then head of health and safety at the British Steel Corporation.

Duncan has also played a part in the academic world. He is currently a visiting professor at the London School of Hygiene and Tropical Medicine, and registrar of the newly-formed Faculty of Occupational Medicine at the Royal College of Physicians. He is a former president of the Society of Occupational Medicine, has a place on the board of the Permanent Commission and International Association of Occupational Health, and is also a member of the Medical Research Council. It was on the subject of relations between the HSE and the MRC that he began his interview with *Nature*.

Duncan recalls that, when Rothschild's "transferred fund system" was implemented, the HSE found itself with some £700,000 to allocate annually to biomedical research. However, unlike the Department of Health and Social Security which was also affected by the Rothschild system, the HSE had few of its own research programmes in this field. It had to "buy itself into research," he says. "HSE's intention was to implement Rothschild by looking at

specific problems. But there was one real difficulty. Very few customers had the necessary expertise to draft research protocols for specific problems." Fortunately, the MRC was on hand to help select suitable projects from the numerous proposals received.

The HSE will have to show that its £700,000 is well spent. If it fails to do so, one result, according to Duncan, will be a loss of trade union support, which could well be disastrous. The commission (HSC) is a tripartite organisation involving the Confederation of British Industry, local authority nominees and the Trades Union Congress. TUC support is obviously vital.

Duncan admits that the HSE places a heavy load on the MRC and that the council is not altogether happy with the arrangement. It is no secret that the MRC would prefer the HSE to follow the example of the DHSS on research work, but Duncan believes that the HSE has yet to reach this stage of development. Relations between the two bodies are reflected in the financial arrangements; until the HSE can assess its own research proposals, it will continue to ask the MRC for guidance, and while this is the case, the MRC has been reassured that most of HSE's research funds will be channelled through its books.

Under the terms of the Health and Safety at Work Act, the onus is on employers to prove the health and safety of each workplace. Duncan sees the HSE's role as monitoring health and safety projects carried out by industry, and cooperating with researchers "to ensure that the work is valid and in no way suspect".

He is deeply disturbed by the conflict in evidence in the United States between scientists in academic circles and those in industry. This polarisation has become so marked that in some cases neither group is prepared to accept the research results produced by the other. Academic scientists have voiced the opinion that the research work of industrial scientists in the field of toxicology is not only biased towards the companies involved, but that results have been deliberately kept secret and in some cases even destroyed. They also claim the research itself is often of poor quality. Scientists in industry have been swift in responding to these accusations. They say the charges may apply to a few companies, but not to the majority. Many have also been heard to complain that scientists in academic circles are making unrealistic and irresponsible demands upon industry for absolute safety.

Should this conflict spread to the UK, Duncan feels one of the most



important functions of the HSE in the next five years could well be as mediator, by ensuring that industrial research, and in particular research into hazards, is of a reputable standard. He also indicates the importance of publishing all results even if they do not yield the "right answers". The health and safety act now makes it compulsory for employers to inform employees about risks (and for the manufacturer, installer and supplier to inform an industrial client similarly).

If the scientific integrity of the researcher could be accepted by all concerned, Duncan believes the conflict need not be so severe in Britain. He refers to the "constant dilemma" which many research workers face when only preliminary results are available. The question everyone asks, he says, is "do you publish the results or withhold the information until you have more evidence to confirm your observations?". Duncan is certain that industry would reveal more information if the response to it was not exaggerated. But he stresses that the one thing the HSE cannot do in this situation is to abet industry in 'covering up' important information. The response of a few trade unions to research on risks is still a crude one; they see it as a potential threat to jobs. Duncan is not enamoured of this response. Ultimately, however, risks will have to be evaluated rationally and this, he suggests, might be a job for the commission.

Duncan sees toxicology as one area in which the HSE will have to offer guidance to the commission for its deliberations. It is recognised that the new HSE notification schemes will generate some friction in industry, and toxicologists will be well to the fore in future controversies. The HSE's intention is to have "plenty of scientific kick" to back up its decisions, says Duncan. And the "kick" will come not only from the ranks of the HSE; the DHSS will be on hand to provide additional support. As a result of this arrangement, Duncan is confident that the executive will be well equipped to cope with the demands put upon it. □



Simsin recording the concentration of respirable airborne dust particles (see p.436).

correspondence

Mincingly speaking

SIR,—My good friend Ken Mellanby (7 September, page 8) fell into the trap of assuming that a word cannot have two meanings. Mincemeat is defined in dictionaries (including the Oxford Dictionary) as either (1) ground, chopped or minced meat, (2) the mixture of raisins, apples, spices etc. used on both sides of the Atlantic in mince pies. The term 'mincemeat' for ground meat may have been abbreviated to 'mince' in Huntingdon—I do not know. But, as a former Sussex man, I recall that Hilaire Belloc said that the Midlands were "sodden and unkind". I would not think of using such language myself, of course.

Yours faithfully,

THOMAS H. JUKES

University of California
Berkeley, CA, USA

mincemeat *n* [alter. of *minced meat*, fr. *minced* (past part. of *mince*) + *meat*] 1: minced meat 2: a finely chopped and usu. cooked mixture of raisins, apples, spices, and other ingredients with or without meat and suet 3: something felt to resemble finely chopped meat; *specif*: a state of destruction or annihilation—used in the phrase *make mincemeat of*: science of making ... of the old-time religion—F.L. Allen: making ... of the inhabitants—Richard Joseph

Geos III not enough for POLO

SIR,—Your articles on the European Space Agency (ESA) (5 October, page 355) are timely as its activities and those of its predecessor (ESRO) have not been widely known to the European scientific community: the present discussions within ESA of future programmes are greatly hampered by this ignorance. However, your statements about the polar orbiting lunar observatory (POLO) need clarifying. You mention the proposal to use the fourth Ariane test flight in 1980 to launch a lunar satellite, building this quickly and cheaply from Geos III hardware. However this is not the POLO concept as only a small part of the necessary complement of experiments to survey the whole surface of the Moon by chemical and physical sensors could be flown on this test flight.

Your report does not make clear that a mission definition study is now being undertaken so that the POLO can be a competitor in the decision as to which mission ESA will launch in 1985. In their recent report ('An approach to long range planning 1980-1990') the ESA Solar System Working Group laid great emphasis on the desirability of European involvement in studies of the Moon and planets and smaller bodies of the solar system, and it is as a result of the interest stimulated by this report that the mission definition study of POLO has been launched. This new development in ESA's thinking recognises that many European laboratories in the last decade have done fundamental work on Apollo and USSR lunar samples, and have participated in planetary missions (see 'The Moon: a new appraisal from laboratory studies and

space missions'. The Royal Society, 1977. Ed. by G. Brown, G. Eglinton, S. K. Runcorn and H. C. Urey). The Ariane rocket has progressed so successfully that Europe will have in the 1980s the technology capable of launching orbiters for scientific study around Mars, the Moon and Venus.

During the preparatory study of POLO, it has been established that there are European laboratories capable of making the experimental hardware for all the various experiments which need to be done (see experiments listed in *Nature*, 265, 197-199 (1977) Runcorn S. K. and Coleman P. J.) but there is now a great need for the European scientific community, particularly young physicists, chemists and geologists, to take these opportunities now unfolding in ESA seriously. Potential interest from European scientists in building experiments or making use of the data to be obtained or perhaps suggesting entirely novel experiments to be done from the orbiter, which the rather closed circle of space scientists may not have envisaged, could be very helpful in the preparatory study now proceeding. (Dr G. Haskall, European Space Agency, 8-10 rue Mario Nikis, 75738 Paris Cedex 15 will supply information.)

Yours faithfully,

S. K. RUNCORN

Department of Physics,
University of Newcastle upon Tyne,
UK.

Chemi-osmosis and energised protons

SIR,—I write this letter to correct some impressions (2 November, page 8) left by your correspondent while describing the achievements of Dr Peter Mitchell for which the Nobel Prize was awarded. Mitchell's distinguished work in 1961 was in the development of a quantitative theory of chemi-osmosis and has been particularly used in explanation of the transduction of energy in biological systems. In this context chemi-osmosis uses a proton gradient. My contribution to this field, to which your correspondent refers most kindly, was to propose first that it was in fact energised protons which were the intermediates in energy transduction. The reference is *The Enzymes*, eds. P. D. Boyer, H. Lardy and K. Myrback, 1, 391; Academic Press 1959.

In 1961 I developed the idea of how energised protons might be used in systems which have diffusion control (*J. Theoret. Biol.* 1, 1; 1961) and one form of this control was independently visualised by Peter Mitchell and myself at that time. This is chemi-osmosis. Where Mitchell and I differed is that he considered this to be the actual mechanism in biological systems. Moreover he developed quantitative tests and did experiments to check his ideas. (It is also fair to add that chemi-osmosis unconnected with energy transduction and unknown to me was developed more

qualitatively by Mitchell during the preceding five years. To be fair to myself, in 1961 before Mitchell's article in *Nature* 191, 141; 1961 was written, he had read both of my papers above).

While Mitchell developed chemi-osmosis I developed an alternative more general approach to diffusion control based on my original concept of energised protons. I think you will understand that in this context I do not accept the concluding remarks by your correspondent about Popper's use of working words. It would be easy to show that Mitchell's ideas and mine could then become lost in the word "chemi-osmosis". Mitchell's theory must be examined in terms of the equations which were derived from it. As I do not accept that chemi-osmosis is a correct description of energy transduction in biology it is very important to keep names tightly associated with ideas. Clearly if in the long term my views are to predominate it is my job to show where chemi-osmosis is incorrect and where my own ideas of localised energised protons are correct. Some papers along these lines have been published and I am about to submit a detailed proposal.

The Nobel Prize has been awarded for chemi-osmosis. In that context it has been very properly awarded to Dr Peter Mitchell and I will be the last to contest this. It so happens that I do not think that it is the correct theory of the way in which energised protons (not originally associated with chemi-osmosis, see above) are used in biological systems. We shall see.

Yours faithfully,

R. J. P. WILLIAMS

Inorganic Chemistry Laboratory,
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US geneticists

SIR,—I wish to make two comments in connection with the article by John Douglas, 'US geneticists look to Europe for research facilities' (21 September, page 170):

● The list of sources of support of the original research leading to the procedure used in synthesising artificial genes should have included the American Cancer Society, not the National Cancer Institute.

● The penultimate paragraph cites the prospective United States patent as at least a partial reason why scientists might be doing certain genetic research in Europe.

The existence of a patent *per se* does not inhibit research in the US or elsewhere. The only basis on which this particular patent (if granted) might become a factor in the choosing of a site of research is that, as noted earlier in the article, industrial licensees of the patent will be required to follow the NIH safety guidelines for genetic engineering.

Yours faithfully,

NIELS J. REIMERS

Office of Technology Licensing,
University of Stanford, USA

news and views

Interstellar carbon

from M. G. Edmunds

SOMETHING in interstellar space absorbs strongly at about 2200 Å. Since the discovery during rocket observations in the early 1960s of a broad absorption feature at this wavelength, more sustained access to the ultraviolet region of the spectrum by satellite observations has shown that the absorber is widespread in our own Galaxy. The detection of this 2200 Å feature in the Large Magellanic Cloud reported on page 478 of this issue of *Nature* by K. Nandy and D. M. Morgan indicates, perhaps not unexpectedly, that the absorber is a common constituent of the interstellar medium of galaxies. But the identification of the material responsible for the absorption remains in dispute. The considerable strength of the feature, normally observed by its influence on the light from a background star, implies that the absorber must be made up of one or more of the most abundant elements. Hydrogen and helium have no suitable features, and the next most abundant elements are oxygen and carbon.

Carbon in either solid or compound form seems to be the most likely candidate, and a similar absorption feature in the theoretical extinction properties of small graphite spheres was noted at the time of the first rocket observations. An elegant explanation of the exact shape of the observed feature was proposed by D. P. Gilra (*Scientific Results from OAO-2*, NASA SP-310, 1972), and this remains the most widely accepted evidence in support of the graphite sphere identification. He suggested that the absorption peak arises from the excitation of a collective mode of oscillation of the electrons, a so-called 'surface plasmon'. The match with observation is very good, provided the particles are very small (of order 0.01 µm in radius) and very spherical. Recent calculations of the expected wavelength dependence of

extinction over both ultraviolet and optical spectral regions for a mix of particles of different sizes and compositions (Mathis *et al.* *Astrophys. J.* **217**, 425; 1977) suggest that the exact size of the graphite spheres is not a very stringent limitation. The 2200 Å feature can still be reproduced by a distribution of graphite particles from 0.005 µm to 1 µm provided that the distribution is heavily weighted to smaller sizes. The fundamental restriction comes from the particle shape, because the wavelength and profile of the absorption feature will change significantly if the particles depart from almost perfect spheres. It may well be that the graphite component of interstellar dust is initially formed in a very spherical shape, or is perhaps subsequently modified to sphericity by collision or erosion. Yet the great anisotropy of the physical properties of graphite would lead to the expectation of a much less symmetrical linear or plate-like crystal growth. Good prospective sites for the formation of graphite dust are known—the carbon-rich atmospheres of some cool stars and the expanding, cooling ejecta of supernova explosions—but so little is known about the growth from vapour phase of small crystals under these low density conditions that no realistic predictions of grain shape can be made.

Other possible culprits for the 2200 Å feature have been proposed, and most of these again involve carbon. The detection at radio wavelengths of fairly large molecules has led F. Hoyle and N. C. Wickramasinghe to propose that a considerable fraction of interstellar carbon is in the form of complex organic molecules. The ultraviolet feature then arises by $\pi \rightarrow \pi^*$ electronic transitions in conjugated carbon bonds (*Nature* **261**, 241; 1976; **270**, 323; 1977). The organic molecules could be incorporated in or on dust particles, and the experimental detection of an absorption hump at 2200 Å in organic extract from the Murchison meteorite is evidence that a feature can be pro-

duced without requiring graphite spheres. The advantage of the bonding argument is that it removes the shape constraints on the dust which carries the molecules. Obviously detailed modelling is required to see if the shape of the observed feature can be accurately reproduced, although the authors admit that preliminary studies indicate that there is probably still a need for some contribution from graphite.

An interesting suggestion that the interstellar carbon may indeed be in solid elemental form, but as the allotrope carbyne rather than graphite, is currently being put forward by A. Webster of the Mullard Radio Astronomy Laboratory. This material (see for example, Whittaker, *Science* **200**, 763; 1978) is extremely difficult to make experimentally, and hence almost nothing is known about its physical properties. Its structure is basically of long parallel chains of conjugated carbon bonds, again giving rise to the hope that it might provide a 2200 Å feature by electronic transitions and independent of particle shape. Quantitative predictions of extinction properties will be required before the relevance of carbyne for the interstellar medium can be assessed. The (admittedly uncertain) phase diagram suggests that it might condense out before graphite in the proposed astrophysical sites of graphite formation, but it is not clear whether it would not subsequently convert to graphite on further cooling and exposure to radiation. Even if carbyne is only important as a short-lived stage in the condensation of graphite dust, this again has implications for the expected graphite dust shape, since the carbyne would probably grow as a linear structure and conversion to graphite is unlikely to produce perfect spheres.

The 2200 Å feature is not the only puzzle in the ultraviolet properties of interstellar material. The continuous extinction in the far ultraviolet considerably exceeds that which would be

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expected from the general extinction of the right amount of small graphite spheres required to match the 2200 Å peak. The calculations on which this conclusion is based rest on classical Mie scattering theory, and there is some evidence (still unexplained) that experimental determinations of the ultraviolet behaviour of such small particles show a much higher extinction than the theoretical prediction (Day & Hufman *Nature* **243**, 50; 1973).

It appears that for the present, despite the qualms about growth shapes, that small graphite spheres remain the best candidate for explaining the 2200 Å absorption. Yet work on alternative explanations is very im-

portant, particularly in the hope that an absorber can be found which not only accounts for this feature, but also for many of the broad shallow absorption features found in the optical spectral region and known as the 'diffuse interstellar bands'. These have been observed for many years, but still defy convincing identification. There is no evidence that graphite spheres would supply such optical features, but on the grounds of simplicity it seems unsatisfactory to invoke yet more components of the interstellar medium to explain them, particularly as additional types of dust must already be proposed to account for the observed continuous extinction over the optical spectrum.

similar ratio for the simultaneous to sequential amplitudes. The non-orthogonality term could not be calculated and was assumed to contribute in the same proportion as in the semi-classical calculation. The overall magnitudes were however smaller, leading to an underestimate of the cross section. This may indicate that the non-orthogonality correction should be smaller in the quantal calculation, or that a more sophisticated treatment of the bound state wavefunctions is needed for simultaneous transfer, as suggested by Feng, Udagawa and Tamura (*Nuclear Phys.* **A274**, 262; 1976).

The general agreement between theory and experiment for two-neutron transfer is in accord with the analysis of Feng *et al.* for calcium targets, and gives more confidence in our understanding of these well matched reactions. Difficulties remain, however, for less well matched reactions which have smaller yields, and for two-proton transfer reactions where theoretical calculations still underestimate experimental results by a large factor. □

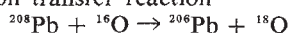
Two-neutron transfer heavy-ion reactions

from P. J. A. Buttle

THE growth in recent years of heavy ion physics, in which target nuclei are bombarded with nuclei accelerated in a heavy ion accelerator, has led to many interesting new phenomena in nuclear physics, but as is often the case with a new field of study, has introduced many new problems. Among the simplest types of reaction which can occur is the transfer of a single nucleon from one nucleus to the other. Apart from a few anomalous cases, these single nucleon transfer reactions are reasonably well understood and it has been possible to obtain useful information about the wavefunctions describing the state of the transferred nucleon in the two nuclei. Reactions in which two nucleons are transferred have been more problematical, theoretical calculations often underestimating the yield of the reactions (or the cross section) by at least an order of magnitude.

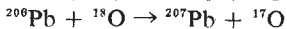
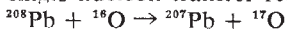
Part at least of the difficulty is that the two nucleons can be transferred either simultaneously or in two separate steps (sequentially), whereas theoretical analyses have often been optimistically confined to only one of these mechanisms. To complicate matters further, the distinction between simultaneous and sequential transfer is not very precise, so that the inclusion of both processes in a calculation involves a certain amount of double counting. Full analysis shows that a 'non-orthogonality' term should be subtracted from the amplitude to allow for this.

A recent careful study of the two neutron transfer reaction



at Minnesota (Franeý *et al. Phys. Rev. Lett.* **41**, 837; 1978) confirms that both simultaneous and sequential transfer are important. The experiments were done at a series of energies low enough (69–73 MeV) that the Coulomb repulsion between the charged nuclei prevents nuclear collisions. The relative motion of the nuclei is then well understood and the transfer of nucleons from one to the other can be investigated without having to face the complexities of a full nuclear collision. The reaction is reasonably 'well matched', that is, the transfer of energy is fairly small so that the classical trajectories followed by the nuclei are not strongly perturbed by the transfer.

The single nucleon transfer reactions



to a series of excited states in ^{207}Pb and ^{17}O were also studied in order that both steps in the sequential transfer be properly understood.

Two types of calculation were reported; the first a semi-classical calculation (Broglia & Winther *Phys. Rept.* **4C**, 153; 1972) in which the relative motion of the nuclei is treated classically. This is a reasonable approximation since the wavelength for classical motion is small compared with the size of the nuclei. Simultaneous, sequential and non-orthogonality terms contribute approximately 50%, 80%, and –30% of the total amplitude, and the result is in excellent agreement with experiments.

The second, fully quantal calculation (second order DWBA) gave a

Pleiotypic effects of hormones

from Robert Shields

LONG before the dawn of molecular biology endocrinologists were aware that steroid hormones exert generalised trophic effects on their target tissues. For instance, oestrogens promote the growth and development of the uterus, glucocorticoids are trophic in the liver and androgens affect male accessory tissues such as the prostate and seminal vesicle. However, these effects are often conveniently forgotten by biologists attempting to understand the action of steroids at the molecular level. Concentrating their efforts on the effects of steroids on a handful of specific proteins such as the egg white proteins of the chick oviduct, workers in this field have made impressive progress culminating in the successful cloning of the ovalbumin gene (Dugai-czyk *et al. Nature* **274**, 328; 1978; Mandel *et al. Cell* **14**, 641; 1978). It will not be long before we understand how steroids act at the molecular level in at least a few restricted cases. The time has now come when the more general (and equally important) trophic effects of steroids should be re-examined.

While the general trophic (or pleio-

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typic) effects of steroids may be the end product of a series of events involving the production of a few new mRNAs (and hence a few new proteins) it seems likely that additional mechanisms are involved. The view that the general trophic effects of steroids and their specific effects on a few proteins may have a different basis is reinforced by the observation that general and specific effects may become separated. For instance, hydrocortisone has a general anabolic effect on the liver and also promotes the production of a specific set of proteins, principally those involved in gluconeogenesis. In hepatoma cells, however, the trophic effect is absent, but the specific effect on the production of a few proteins including tyrosine transaminase is still present (Ivarie & O'Farrell *Cell* **13**, 41; 1978). Until recently there were few well-studied cases of the reverse situation where the general trophic effect of steroids could be observed without concomitant specific effects. Recent work on the rat seminal vesicle suggests that this may be a good system to do just that.

About 30% of the protein synthesised in the rat seminal vesicle is accounted for by two secreted glycoproteins of the seminal plasma. The synthesis of these proteins is controlled by testosterone since their synthetic rates fall after castration and rise after hormone treatment (Higgins *et al.* *Biochem. J.* **158**, 271; 1976). So far this looks just like another case of specific protein induction by steroid hormones. Where these results are interesting is that the synthetic rates of these proteins follow the overall rate of protein synthesis which in turn parallels total mRNA levels (Higgins & Burchell *Biochem. J.* **174**, 543; 1978). So in this case the rise (or fall) of the levels of the mRNAs of these two proteins is not a consequence of the specific rise (or fall) of their respective mRNAs (as is the case with ovalbumin for instance) but is a result of some general change in metabolism of the entire spectrum of mRNAs in the tissue. Careful analysis of the sequence complexity of the mRNAs isolated from the tissue in different hormonal states confirms this conclusion (Higgins *et al.* *Eur. J. Biochem.* in the press) and furthermore, these changes in total mRNA levels also parallel changes in the total RNA content of the organ (Higgins & Burchell *Biochem. J.* *op. cit.*). Since mRNA accounts for only a fraction of the total RNA in the cell this result shows that in this tissue at least, androgens may regulate the overall levels of RNA transcription rather than the transcription of a severely limited number of genes.

These results are strong evidence that androgens act to produce the

trophic response in the seminal vesicle without inducing new mRNA species; however, in other tissues androgens are capable of inducing new proteins. In the ventral prostate, for instance, androgens control the synthesis of four major proteins that, like the seminal vesicle proteins, are glycoproteins secreted into the seminal plasma (Hayns *FEBS Lett* **81**, 43; 1977; Parker *et al. Biochem. J.* **170**, 115; 1978). In distinct contrast to the situation in the seminal vesicle these proteins are not regulated by general changes in overall mRNA synthesis but rather by specific effects on their individual genes (Parker & Mainwaring *Cell* **12**, 401; 1977; Hayns *et al. Biochem. biophys. Res. Commun.* **77**, 1492; 1977; Parker & Scrae *Eur. J. Biochem.* **85**, 399; 1978).

These results show that alterations in the synthesis of major secretory proteins may be brought about by two different means, one is the (dare one say) more traditional alteration in the levels of specific mRNAs coding for those proteins, and the other is through modulation of the production of RNA as a whole. Quite why these two different strategies are adopted for the same hormone in functionally closely related tissues of the same animal is however far from clear. □

Amorphous silicon

from a Correspondent

THE letter published in this issue of *Nature* (page 482) by S. R. Ovshinsky and A. Madan highlights the intense activity in laboratories all over the world seeking to exploit the potentialities of amorphous silicon for photovoltaic solar cells. For a solar cell which does not make use of lenses or mirrors to concentrate the Sun's radiation, it is obviously necessary to cover a very large area, and the outstanding advantage of deposited films of amorphous silicon is their cheapness, compared with the single crystals of the usual silicon technology.

The possibility of using amorphous silicon is a very recent development. It is only 15 to 20 years since it was recognised that glasses and amorphous materials generally have conduction and valence bands very similar to those in crystals. The early work of Kolomiets and coworkers in Leningrad on the semiconducting chalcogenide glasses, for instance As_2Te_3 , showed that, unlike crystals, these glassy semiconductors cannot be doped. The reason seems to be that in a glass any atom takes up a configuration such that all its outer electrons form bonds with nearest neighbours. Thus As has three

neighbours in the glass, Te two and if Ge is added we expect it to have four. Amorphous silicon is not of course a glass, in the sense that it cannot be prepared from the melt; but films prepared by deposition have the normal fourfold coordinations and behave as semiconductors. Properties of evaporated or sputtered films are not the same as those that would be expected from a continuous random network, in which every atom is bonded to four neighbours. For instance, electron paramagnetic resonance investigations reveal a high concentration of unpaired spins, presumably located at dangling bonds occurring on silicon atoms bonded to only three neighbours. Moreover these and other places where the continuous random network is broken produce what are called 'deep states in the gap', that is to say points where an electron may be trapped with a value of its energy somewhere midway between the conduction and valence bands. Such states, as also in crystalline semiconductors, act as recombination centres; if an electron and the corresponding positive hole are formed by the absorption of radiation, they recombine much faster in the neighbourhood of one of these deep states, which allows the electron to get rid of its energy in two steps. In fact, so rapid is the recombination in evaporated films that it is difficult to observe a photocurrent.

The Dundee school under W. Spear, which included at that time the junior author of the paper under discussion, showed that in films prepared by the deposition of silane (SiH_4) from a flow discharge, the number of these deep states could be very much reduced, so that a significant photocurrent could be observed. There has been some dispute about why this is so but it now seems reasonably certain that, instead of dangling bonds, Si-H bonds are formed, which do not give rise to states in the gap. The films appear to contain 5% of hydrogen or more and if the hydrogen is introduced in other ways the properties are similar. Some deep states, however, remain; there has been much speculation as to their nature, and also much effort to reduce their number, so as to reduce the rate of recombination.

Now the paper by Ovshinsky and Madan claims that fluorine will do all that hydrogen did and more; it reduces the number of states in the gap considerably more than hydrogen does, and gives greater stability. The importance of this lies in the discovery by the Dundee group a few years ago that, unlike Kolomiets' glasses, one can dope hydrogenated amorphous silicon, for instance by adding phosphine to the silane. Some, but not all, of the phosphorus appears to go in with fourfold

coordination, and this produces donors.

It has not yet proved possible to incorporate a concentration of donors greater than the concentrations of states in the gap, and therefore the donors lose all their electrons to these states. But the Fermi energy is thereby raised and can be brought quite near to the bottom of the conduction band. And similarly, by doping with boron, it can be brought near to the edge of the valence band. It is thus possible to produce a p-n junction, or a Schottky barrier, and thus a photovoltaic cell.

From these results from Ovshinsky's laboratory, it seems then that this can be done with less doping and greater stability by replacing hydrogen by fluorine. This may turn out to be quite an important step on the long road to an efficient solar cell. □

Vertebrate skin

from R. I. C. Spearman

THE successful investigation of any organ system involves many different disciplines. This was especially evident at a recent symposium on the 'Skin of Vertebrates'* held recently in London.

R. J. Roberts (University of Stirling) and A. Bullock (Scottish Marine Biological Association, Oban) have found that in teleost fish the epidermal cells are mitotically active at all levels. This is in contrast to mammals, in which cell division is normally limited to the basal layer. In consequence, skin wound healing in fish occurs without the need for a preliminary burst of mitotic activity as seen in mammalian epidermis.

A. M. Lucas (Michigan State University) and A. G. Matoltsy (State University, Boston) showed that chicken epidermis is peculiar in that appreciable free lipids are endosecreted into the cytoplasm in addition to keratin. In contrast, mammalian epidermal cells produce little free lipid except in the sebaceous glands. R. Wrench (University College Hospital Medical School, London) has found extensive cytolysis in chicken epidermis as in sebaceous glands and she suggested that chicken epidermis is intermediate in type between the keratin-forming mammalian epidermis and lipid-secreting skin glands.

Studies on the supramolecular structure of the hair keratin complex which is constructed from many different proteins have made major advances in the past few years with long amino-acid sequences now known for some of

the constituent proteins. For many years it had been thought that disulphide linkages stabilise keratins by forming cross-links between protein chains. However, R. D. B. Fraser (CSIRO, Melbourne) pointed out that there now seem to be very few disulphide cross linkages and most disulphide bonds link different points on the same chain. Nevertheless, reducing agents which break these intra-chain bonds, such as depilatory agents, soften hair keratins. How such intrachain linkages can stabilise arrangements between adjacent protein chains is not clear, but is of importance in the bonding of high molecular substances in general.

Since the turn of the century it has been known that the nerves to a tissue have some stimulatory effect on cell growth. This was first noticed in cases of gunshot wounds in which the severance of nerves to the hand had slowed down the rate of nail growth. The work of M. Singer (Case Western University, Ohio) and his colleagues on the action of nerves on limb regeneration in amphibia showed that both motor and sensory neurones promote epidermal cell division, RNA transcription and protein synthesis. The active agent has been found to be a protein which has been isolated from nerves.

Both fetal skin differentiation and adult modulation depend on interactions between dermis and epidermis. This has been studied using recombinants of dermis and epidermis from different sites. P. F. Goetinck (Connecticut State University) has found that some genes for keratinised structures act on the dermal cell transmitter of the inductor stimulus and others on the epidermal cell receiver. P. Sengel (University of Grenoble) showed that in embryonic life both the regional epidermal morphology and the particular keratin proteins formed by epidermal cells are dependent on induction by dermal cells. This is even true of recombinants between fetal mouse epidermis and chicken dermis, and so the ability of dermal cells to control epidermal functions can be transmitted across zoological class barriers. The epidermis retains some intrinsic rhythmic activity as in the sloughing cycle of snakes and lizards.

The work of A. W. Poole (Rutgers University, New Jersey) also using tissue recombinant methods has shown numerous interactions between melanocytes and epidermal cells of the hair follicle. Thus, some mutant genes which alter coat coloration act directly through the melanocytes and others act on the hair formative cells to modify the melanin pigmentation. D. T. Parkin (University of Nottingham) studied pigmentation polymorphism from another viewpoint; the effects of ecology and sexual selection on population genetics.

A good example of this is the changing pigmentary patterns of pigeons seen in city streets.

As A. Iggo (Royal Dick School of Veterinary Studies, Edinburgh) pointed out in a final session devoted to neural sensation, to understand the functioning of a sensory receptor it is necessary to study the electrical activity of an efferent nerve in response to different specific stimuli, which must then be correlated with histological findings. There is good evidence that the mammalian Pacinian corpuscle and the Meissner corpuscle are pressure and touch receptors respectively, and the fact that separate sensory areas in the brain are present for heat and cold would indicate special receptors for thermal senses, although the physiological work on these is incomplete. The suggestion put forward some 20 years ago that there are no specific sensory receptors has prompted extensive research on the subject by those who believe in specificity. However, various speakers showed that in all vertebrate classes microscopy of sensory receptors has greatly outstripped physiology, and it will be some years before we fully understand skin sensation, even in mammals. □

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Accuracy in the transmission of biological information

from a Correspondent

THE 'recognition' of one biological molecule by another has long been acknowledged to be one of the fundamental physical principles of biology. In all cases the recognition is a first step, preceding a subsequent reaction in which the correct substrate reacts further along a path from which incorrect substrates must be excluded.

More recently it has become possible to describe in quantitative terms the correctness of the recognition using an error level parameter, which expresses the ratio of the numbers of correct and incorrect substrates reacting. The error level can in many cases be measured, for example by offering *in vitro* the correct and incorrect substrate in equal amounts and examining the formation of correct and incorrect products. Typical error levels are, for protein synthesis from mRNA, 1 in 10⁴ (one

*EMBO Workshop, 'Accuracy', was held in Grignon, France, on 12-15 September, 1978 and organised by Dr Jacques Ninio.

*Held on 25-27 September and organised by the Linnean Society of London in conjunction with the British Association of Dermatologists. The Proceedings will be published for the Linnean Society by Academic Press.

wrong amino acid incorporated for every 10^4 right ones) and for DNA replication 1 in 3×10^4 . *In vivo* the error level in protein synthesis can be measured by observing a mutant in which the gene for a particular enzyme is blocked by a nonsense mutation; the appearance of small amounts of this enzyme in spite of the mutation gives a measure of the rate at which the nonsense is misread as sense.

Error levels found *in vivo* are often lower than those found *in vitro* which means that (neglecting artefacts of extraction) the organism can and does reduce its error level by increasing the complexity of the recognition process—spontaneous mutation rates fall in the range of 1 wrong base for every 10^7 to 10^{11} right ones. The error levels expected can also be predicted thermodynamically by appeal to the relationship between order and entropy: the selection of a correct substrate rather than a random one involves an increase in the amount of order in the biological system and is therefore thermodynamically unfavourable, unless other factors can be brought into play. These factors are, in part, well known: the Watson-Crick interaction in DNA replication, surface complementarity between enzyme and substrate, codon-anticodon binding on the ribosome. Since the energy involved in the selection is often known (for example, the difference between the energies of formation of a correctly-matched and a mismatched Watson-Crick pair) the error level can be predicted using simple thermodynamics. Such predicted error levels are very frequently orders of magnitude higher (that is, worse) than those achieved in cells, demonstrating yet another feat of nature which chemists can at present neither reproduce nor properly explain.

In the hope that this problem might, however, yield one day to the combined onslaught of interested mathematicians, physicists, chemists, biochemists and biologists, a meeting on 'Accuracy' was held recently*. Of principal interest were problems such as those outlined above—DNA polymerisation, protein synthesis, codon recognition, information and its relation to thermodynamics and kinetics—but the meeting also included related topics such as antibody specificity, accuracy and evolution, protein degradation and ageing.

The greatest interest was aroused by subjects relating to the central problem of molecular biology—the transmission of hereditary information over many generations of DNA and the realisation of this information in the synthesis of protein. To explain the low error levels seen in these processes, the idea of 'proofreading', has been postulated by Ninio and Hopfield (Ninio *J. molec.*

Bronze Age honey

from Peter D. Moore

THE analysis of pollen grains from samples of honey is now a commonly used technique for determining what plants are used by foraging bees. It has recently been put to a rather unexpected use by Dickson (*Antiquity* 52, 108; 1978) in the analysis of materials from a Scottish Bronze Age barrow at Ashgrove, Fife.

Within a burial cist a large quantity of decomposed plant material was found, containing the fruits of birch and seeds of rushes, together with mosses including *Sphagnum*. Over the skeleton there was a deposit of black crumbly material which, when subjected to analysis, was found to contain pollen in the proportions 54% lime (*Tilia*), 15% meadowsweet (*Filipendula*), 8% heather (*Calluna*) and 7% plantain (*Plantago lanceolata*) as well as various other minor components. The very high values for lime are exceptional, for this tree is insect pollinated. It is therefore unlikely that this assemblage represents the contemporaneous pollen fallout when the burial took place.

Dickson considers two possible explanations. It is conceivable that flowers were placed within the tomb and, since lime and meadowsweet both flower in July, their presence together is reasonable. Glob (*The Mound People*, 61, Faber and Faber, 1970) describes the use of flowers in the Bronze Age burials of Denmark. Yarrow (*Achillea millefolium*) was

found in an oak coffin and this plant was used extensively both for foretelling the future and for healing wounds. No such significance is attached to lime or meadowsweet.

An alternative explanation, which Dickson favours, is that an overturned beaker also found in the burial may have contained honey or mead which had originally been produced from lime flowers. This would also explain the presence of meadowsweet (medesweete) which was widely used as an additive to mead. Dickson has found support for his argument in the analysis of scrapings from the outside and inside surfaces of the beaker. Only the inside contained lime and meadowsweet pollen, the greatest concentration being upon the lower surface of the tipped vessel. It therefore seems certain that the beaker contained honey or a honey derivative, making it the oldest record of honey in Britain, with a radiocarbon date of 3046 ± 150 b.p.

Dickson is inclined to believe that the honey was imported either from England, where lime was a far commoner tree in Bronze Age times (judging from the level of its pollen in peats and lake sediments) or even from Denmark, where Bronze Age honey has previously been recorded. There is, however, no firm evidence upon which to come to a conclusion. A single lime tree in the vicinity of a hive could account for the pollen assemblage, and lime would be capable of flowering in Fife even if it could not set ripe fruit. So the possibility still remains that this first Scottish honey was home produced.

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Biol. 84, 297; 1974; Hopfield *Proc. natn. Acad. Sci. U.S.A.* 71, 4135; 1974). They have suggested that in cases where a low error level is important the bound substrate is double-checked in a second recognition step, and rejected if it is the wrong one.

Proofreading may take place in many ways, so that according to C. Kurland (Uppsala) "we now have more theories to explain accuracy in protein synthesis than experimental results to test them." However, the proofreading theory does make falsifiable predictions, one of which is that the offer of a great excess of wrong substrate should result in a high turnover of the first recognition step, with rejection of the wrong product at the second. This has been observed by R. Thompson (University of Pennsylvania, Philadelphia) who compared the amounts of

energy-rich triphosphate consumed when an artificial mRNA was used in the synthesis of the correct or (under forcing conditions) the incorrect polypeptide. For every incorrect amino acid incorporated around 30 triphosphate molecules were hydrolysed, implying that this number of incorrect amino acid molecules is eliminated at the second recognition (proofreading) step. The incorporation of the correct amino acid, which is not rejected at the proofreading step, proceeds of course much more rapidly and less wastefully.

Other forms of proofreading postulated include one by F. von der Haar and F. Cramer (University of Göttingen), in which the chemical environment of the recognition site enhances the accuracy of the recognition ('chemical proofreading'). In

another, postulated by A. R. Fersht (Imperial College, London) the amino acylation site rejects competing amino acids which are larger than the correct substrate but accepts smaller amino acids. These pass to a second, hydrolytic site which, again on steric grounds, rejects the correct substrate but destroys the reaction product formed with the competing smaller amino acids.

This mechanism has been dubbed 'double sieve editing'. R. Mulvey (MRC Laboratory of Molecular Biology, Cambridge) presented results which indicate that this is how, for example, valyl-tRNA synthetase rejects with such efficiency competitors which differ merely by one methylene group from the correct substrate, valine.

If the occurrence of proofreading can be finally confirmed, then future work in this field will clearly include the search for non-proofreading mutants and for a molecular description of the proofreading process itself, which, in spite of its complexity, must have originated at a very early stage in the evolution of the cell. □

Carcinogen binding to DNA

from Stephen Neidle

It is now generally acknowledged that the polycyclic aromatic hydrocarbons are not directly carcinogenic but are metabolised *in vivo* to short-lived reactive species that bind to cellular macromolecules, initiating cancerous processes in some way. Most evidence indicates that DNA is the critical receptor—a conclusion strengthened by the relationship between mutagenicity and carcinogenicity indicated by 'Ames-type' bacterial test systems. Over the past few years much activity has been directed at understanding the chemistry of the interactions of such metabolites with DNA. But why such events are carcinogenic remains unknown.

In the case of benzo[a]pyrene, an important metabolite seems to be the *trans* isomer of its 7,8-diol,9,10-epoxide derivative; this electrophilic compound has far greater mutagenic and carcinogenic activity than other isomers. It binds predominantly to guanine residues in DNA, with the N2 position of the purine base being thought to be a major site of attack. (However, this picture is complicated by the recent

finding of Osborne, Harvey & Brookes (*Chem.-Biol. Interactions* **20**, 123; 1978) that the N7 atom of guanine is also susceptible to attack by the diol epoxide; the instability of this adduct explains why it was not detected in previous studies).

Identification of likely sites on DNA for covalent attachment of a carcinogenic molecule is a vital prerequisite for any attempt at visualisation of the conformational and structural changes induced in the DNA. Grunberger, Weinstein *et al.* have utilised such information in an examination (by means of circular dichroism spectroscopy) of the conformational properties of the dinucleoside phosphate GpU to which was bound benzo[a]pyrene diol epoxide, at the N2 position of the guanine base (*Biochemistry* **17**, 1278; 1978). GpU was considered to be a model for a nucleic acid; however this can only be true in a restricted sense since it cannot base-pair and thus form a fragment of double-stranded polymer. Nevertheless, the finding that the bound polycyclic hydrocarbon interacts in some (probably stacking interaction) manner with the uracil residue, is of interest and some significance. As Grunberger *et al.* point out, such an interaction profoundly alters the conformation of the molecule around the guanine-sugar linkage, such that if it were in a double strand the guanine would be forced out of its base-pairing position. Grunberger, Weinstein *et al.* have previously shown (*Biochemistry* **16**, 3127; 1977) that benzo[a]pyrene diol epoxide-modified DNA has small local regions of denaturation and instability, as instanced by increased sensitivity to S_1 nuclease attack relative to native DNA.

These molecular concepts have now been taken a step further by Drinkwater, Miller and Yang (*Cancer Res.* **38**, 3247; 1978) who have examined the binding and mutagenic behaviour of a number of aromatic electrophilic carcinogens. These ranged from the potent benzo[a]pyrene diol epoxide to simple ethylene oxide-aromatic derivatives. Reasoning that the planar aromatic groups common to such molecules could well intercalate into DNA (an old, but hitherto largely discredited theory), Drinkwater *et al.* measured the unwinding induced in closed circular supercoiled SV40 DNA. This technique is widely used as a tool in the investigation of known intercalative drugs such as ethidium bromide, proflavine and actinomycin. Unwinding and consequent reversal of supercoiling is considered to be diagnostic for intercalative binding. Such an effect was observed for almost all the compounds studied, although with striking differences in unwinding activity between the diol epoxide and 1-pyrenyloxirane

on the one hand, and such compounds as 7-benzanthryloxirane on the other. Interestingly, a strong positive correlation was found between unwinding ability, and frame-shift mutagenic activity (in a *Salmonella* Ames test) for the range of compounds tested. Overall, the results of Drinkwater *et al.* clearly point to local unwinding of DNA being the consequence of the covalent binding of an active compound. They have used this information as the basis for a plausible molecular model for the structure of the DNA adduct. In agreement with the notion of local denaturation at the binding site, the residue to which for example, benzo[a]pyrene diol epoxide is bound (be it at the N2, N7 or even C8 positions), becomes destacked from its adjacent bases and thus protrudes to some extent. The aromatic part of the carcinogen is postulated to intercalate between the adjacent bases, in a similar way to the non-covalently-bound intercalating drugs. Confirmation of this model in detail must await a definitive X-ray structure analysis of, say, a carcinogen-deoxyoligonucleotide complex. The visualisation of drug-nucleic acid binding in this way is currently a topic of lively interest; it is to be hoped however that in the case of covalently-bound carcinogens, the pitfalls of extrapolating from relatively simple model complexes to polymers, will be adequately perceived.

That guanine residues are the major target in DNA for diol epoxide attack, is now well established; however what is rather less clear is whether such bindings themselves are the major carcinogenic events, or indeed what relationship they have to such events. The complexity of the situation is emphasised by the recent work of Kakefuda and Yamamoto (*Proc. natn. Acad. Sci. U.S.A.* **75**, 415; 1978) who have also examined benzo[a]pyrene diol epoxide modification of DNA. They, like Drinkwater *et al.* observed local unwinding of closed circular DNA. However, analysis of the S_1 nuclease-treated product showed that predominantly adenine, and not guanine residues, were modified so as to cause local unwinding-induced denaturation, even though the latter bases were the major sites for actual binding of carcinogen. Furthermore, examination of double-stranded synthetic polynucleotide-carcinogen complexes showed that only the A-T one was S_1 sensitive. Kakefuda and Yamamoto thus suggest that there are (at least) two categories of base modification; adenine involvement producing local unwinding and base-pair cleavage, and guanine attachment, with an altogether less drastic resultant. However, the general features of the molecular model proposed by Drink-

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water *et al.* are unaffected by any such controversies concerning the precise nature of the carcinogenic binding site, and so may well be found to be roughly correct. □

The sequence of *E. coli* 16S ribosomal RNA

from Richard Brimacombe

THE development of rapid gel techniques for sequencing nucleic acids has provoked a scramble to finalise the sequences of the *E. coli* ribosomal RNA molecules. Several research groups have taken part in this race, and two complete sequences of the 16S RNA have just been published. Pride of place goes to the Californian group (Brosius, Palmer, Kennedy & Noller, *Proc. natn. Acad. Sci. U.S.A.* **75**, 4801; 1978) whose sequence was determined from a plasmid DNA containing one of the 16S ribosomal RNA genes, using the DNA techniques of Sanger and Coulson (*J. molec. Biol.* **94**, 441; 1975) and of Maxam and Gilbert (*Proc. natn. Acad. Sci. U.S.A.* **74**, 560; 1977). This sequence was first reported at the EMBO ribosome workshop in Salamanca in June (see *News and Views* **247**, 743; 1978), and it has been closely followed into print by a complete sequence from the Strasbourg group who presented a partial sequence based on gel data in Salamanca, which has since been finalised (Carbon, Ehresmann, Ehresmann & Ebel, *FEBS Lett.* **94**, 152; 1978).

This latter sequence, obtained entirely independently and without so much as a mention of the Californian group, is derived from a direct analysis of RNA-fragments, using the technique first applied to a polyribonucleotide by Donis-Keller *et al.* (*Nucleic Acids Res.* **4**, 2527 (1977)).

Publication of these two sequences closes a subject which has been a matter of controversy for some years. Work on the 16S sequence using classical techniques was initiated by the Strasbourg group about 10 years ago, and led, after some preliminary papers, to the publication of a detailed oligonucleotide catalogue (Fellner *et al.* *Biochimie* **54**, 853; 1972) and to a first tentative sequence of the molecule (Ehresmann *et al.* *Biochimie* **54**, 901; 1972). However, two years later a

paper appeared from Woese's group in Illinois (Uchida *et al.* *J. molec. Evol.* **3**, 63; 1974) which claimed that over 50% of the ribonuclease T₁ sequences published by the Strasbourg group for oligonucleotides of eight or more bases in length were incorrect, and this was shortly followed by a second confirmatory paper by the same group (Magrum *et al.* *Nature* **257**, 423; 1975). At about this time the Strasbourgers went to press with a new version of the 16S sequence (Ehresmann *et al.* *Biochimie* **57**, 711; 1975). This sequence, which claimed to be 90–95% complete, was very considerably modified with respect to the 1972 version, and some but by no means all of the T₁-oligonucleotide sequences were altered to agree with those of Woese's group. At this stage about 25% of the T₁-oligonucleotides remained to be finally ordered within the sequence, but there were also queries being raised concerning some of the existing orderings (see for example Noller *Biochemistry* **13**, 4694; 1974).

The next version of the Strasbourg sequence appeared in 1977 (Ehresmann *et al.* *FEBS Lett.* **84**, 1977), and contained a number of substantial improvements. Additional information was given for nucleotides in regions totalling over 300 bases, the number of T₁-oligonucleotides remaining not finally ordered was reduced to a total of just over 200 bases, and almost all of the T₁-oligonucleotide sequences were in agreement with Woese's data. At this stage, recognising that application of the classical methodology was becoming subject to a law of rapidly diminishing returns, the Strasbourg group turned their attention to gel sequencing technology, and have now completed the sequence as quoted above. In this final version, all the T₁-oligonucleotides are ordered and in addition there are alterations to sequences involving a total of almost 200 bases with respect of the 1977 data, which of course brings us to the crucial question—how well does this latest RNA sequence agree with the DNA sequence obtained by Noller's group? And the answer is, the agreement is excellent. The Californian sequence is 1,541 bases long, the Strasbourg sequence 1,542 bases, and within this whole length there are (by my count) only eight single-base discrepancies. The data agree also with sequences at the 3' and 5' ends of the molecule obtained by Young and Steitz (*Proc. natn. Acad. Sci. U.S.A.* **75**, 3593; 1978), and all of the Woese T₁-oligonucleotide sequences (with one possible exception, containing one of the eight above-mentioned disputed bases) are correct. So everybody should be happy, and the way is now clear for serious attempts to be made to determine the secondary

and tertiary structure of the molecule.

It is perhaps worthwhile to consider very briefly here the relative merits of the DNA compared with the RNA gel methodology for the sequencing of long RNA molecules. Aside from the purely technical viewpoint (where any comparison of the two sets of sequencing data is difficult, since the Strasbourg publication contains no experimental documentation), there are several points to consider. First, the DNA method gives the sequence of a single cistron, whereas the RNA method gives the 'average' of all the cistrons for the RNA molecule concerned; this undoubtedly accounts for the small but significant number of heterogeneities observed in the 16S RNA by the Strasbourg group. Second, the DNA method has an obvious advantage in allowing the use of restriction enzymes to generate the fragments for sequencing; in the RNA method, the production of suitable fragments is a rather hit-and-miss affair, although here it is noteworthy that the Strasbourg group have for this purpose made good use of the double-strand-specific cobra venom nuclease of Vasilenko and Rytte (*Biokhimiya* **40**, 578; 1975). Third, while the DNA method is for this last reason certainly the cleanest and quickest for sequencing the whole molecule (once the appropriate cistron has been isolated), the RNA method is likely to prove most useful for people working with ribosomes or similar particles and wanting a rapid analysis of a particular region of the structure. Finally, it should not be forgotten that the classical methods of oligonucleotide analysis are still needed, both to establish the nature and location of any post-transcriptional modifications such as methylation, and—particularly in the case of the RNA gel method, as has been clearly shown in a similar sequencing study by Gross *et al.* (*Nature* **273**, 203; 1978)—to confirm the sequence. □

Meteorology over the tropical oceans

from D. B. Shaw

A CONFERENCE on meteorology over the tropical oceans was held in London during August this year.* By its very nature, meteorology is a science requiring international cooperation and it was fitting that the conference was jointly arranged by the Royal

*The proceedings of the conference, in which the current state of the science is reviewed by ten leading authorities in their fields, are to be published early in 1979 by the Royal Meteorological Society, James Glaisier House, Grenville Place, Bracknell, Berkshire.

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Meteorological Society, the American Meteorological Society, the Deutsche Meteorologische Gesellschaft and the Royal Society. One of the main purposes of the conference was to provide a platform for the presentation of research related to GATE, the GARP (Global Atmospheric Research Programme) Atlantic Tropical Experiment. The field phase of GATE was completed in 1974, and has been described by Mason (*Nature* 255, 17; 1975). Since then various designated data centres have been processing the data to make them available to the meteorological community at large. Much of that work is now completed and many research projects making use of the data are now underway. The conference provided an opportunity to assess the progress that has been made in tropical meteorology in the past few years and to identify the outstanding problems.

Central to the purpose of GATE was the problem of scale interaction—the way in which convection, which is a vital heat transfer process in low latitudes, acts on and reacts to the larger scale flows of the tropical atmosphere. A fundamental obstacle in understanding this interaction is the relatively small scale of convective processes. Deep penetrative convective towers occupy only a tiny fraction (about 1%) of the tropical atmosphere. The standard observational network cannot hope to give an adequate account of such small scales of motion. They also present a difficulty to the numerical modeller. Most numerical models of the atmosphere have a spatial resolution too coarse to resolve convective processes explicitly. Their effects are simulated, or parameterised, by idealised formulations which are designed to represent the effect, not of an individual convective cell, but of the hypothetical ensemble of cells acting in the region represented by each of the model's grid points.

As a result of GATE the meteorologist now has, for the first time, a fairly detailed description of the atmosphere on scales from the synoptic down to the convective, for part of the tropics at least. The three-dimensional structure of the large scale (~2500 km) travelling disturbances over West Africa and the East Atlantic has now been well documented. These easterly waves, usually found in the months from June to October, have associated with them marked convective activity which is generally found in the region ahead and slightly to the south of the vorticity centre of the disturbance. From a consideration of energy exchange processes it can be deduced that the latent heat release in the convection plays an important part in the develop-

ment of the wave over West Africa. The role of convection later in the life cycle of the disturbance (that is, over the ocean) is less clear. Of course the convective mechanism is not to be seen purely as a forcing action on the larger scale flow; the convection is itself responding to conditions of the large scale flow.

Hurricanes that occur in the Caribbean can sometimes be seen to develop from the easterly waves with their origins over West Africa. In terms of maintenance of the atmospheric global circulation hurricanes are thought not to have a major role. They do, however, have a large local impact and are the most destructive type of weather system. An examination of hurricane records for the past 20 years reveals interesting features. Two thirds of all tropical cyclones form in the northern hemisphere. Tropical cyclones, which may or may not develop full hurricane strength, do not form in the immediate vicinity of the equator, the latitude belts 5°–15° being the preferred regions. In the majority of cases their development is very close to the Inter Tropical Convergence Zone. Their temporal distribution shows preferred periods of development, not just in seasonal terms but on a time scale of 10–20 days, probably in response to

variations on the same time scale in the large scale tropical circulation. The specific criteria which influence development of an individual cyclone are seen to be a combination of thermal and dynamical conditions. Thermal instability in the vertical is not the *sine qua non* for cyclone development. Low level cyclonic circulation—and an upper tropospheric counter circulation—are seen to be particularly relevant to development.

The use of numerical models of the atmosphere for weather prediction is now commonplace in many national meteorological services outside the tropics. The application of such models in the tropics is more problematical because of the subtle nature of the balance between wind and pressure fields in equatorial regions. However, using initial conditions derived from GATE data, encouragingly good numerical forecasts out to a few days have been made by groups in both the USA and the UK. It would seem that such forecasts have similar quality, in terms of wave movement and rainfall patterns, as the operational models of middle latitudes. The extent to which parameterisations used in such models are capable of adequately representing sub grid scale processes remains an open question. □



A hundred years ago

AMERICAN JURASSIC DINOSAURS.—Prof. O. C. Marsh publishes in the current number (November) of the *American Journal of Science and Arts* the principal characters of some new species of dinosaurs. On the flanks of the Rocky Mountains a narrow belt can be traced for several hundred miles, which is always marked by the bones of gigantic dinosaurs. The strata consist mainly of estuary deposits of shale and sandstone, and the horizon is clearly upper Jurassic; the dinosaurian remains in this series of strata are mostly of enormous size, and indicate the largest land animals hitherto known. One new species (*Atlantosaurus immanis*) must have been at least eighty feet in length and several others nearly equalled it in bulk. With these monsters occur the most diminutive dinosaurs yet found, one (*Nanosaurus*) not being larger than a cat. Some of these new forms differ so widely from typical dinosauria that Prof. Marsh has established a new sub-order to receive them, called *Sauropoda*, from the general character of the feet. They are the least specialised forms of the order, and in some of their characters show such an approach to the mesozoic crocodiles as to suggest a common ancestry at no very remote period. In

them the front and hind limbs are nearly equal in size; the feet are plantigrade with five toes on each foot. The carpal and tarsal bones are distinct; the precaudal vertebrae contain large, apparently pneumatic cavities; the sacral vertebrae do not exceed four, and each supports its own transverse process. The pelvic bones unite in front by a ventral symphysis; the limb bones are solid. One of the species described and partly figured in Prof. Marsh's paper is called *Morosaurus grandis*; when alive it was about forty feet in length; it walked on all four legs, was probably very sluggish in its movements, and had a brain proportionally smaller than any known vertebrate.

PROF. WURTZ was charged some time since by the French Minister of Public Instruction, to make an inquiry into the organisation of the laboratories and practical instruction given in the several universities of Germany and Austro-Hungary. Prof. Wurtz accordingly made several journeys to the great seats of learning in these two countries, and the *Journal Officiel* of last Saturday publishes at full length his report. Prof. Wurtz insists strongly on the danger of creating large establishments, where students are taught something of everything, and on the necessity of creating special foci for every large section of experimental science. He shows the advantage of special institutes, and insists upon the organisation of chemical, physical, physiological, anatomical, and pathological institutions such as flourish on the other side of the Rhine.

From *Nature* 19, 28 November, 76, 87; 1878.

review article

A diagram of evolution

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Recent developments in molecular genetics have made it possible to re-examine the question of the relationship between chromosomes and organisms, and heredity and evolution, in the light of what is now known about the molecular basis of all of them.

OUR methods of studying chromosomes effectively and of breeding organisms experimentally were discovered by separate paths and only later did the two kinds of enquiry combine to reveal the bases of heredity and evolution. For half a century they had proceeded in parallel resting on different assumptions and implying a different understanding of the subject which was now to be called genetics. Thus in 1932 when I came to summarise what was known of the chromosomes I was able to argue a convincing unity in the subject. But beneath this unity I was forced to acknowledge a conflict which the chromosome theory was thought to have excluded. For the chromosomes were seen to have evolved according to rules of their own not clearly related to any properties of the organisms whose heredity they were supposed to be carrying¹.

The development of genetics in the past 40 years has confirmed the unity of the subject but it has scarcely noticed this conflict within it. Over the same period, however, there has been added to the old genetics a new genetics. The microbial materials and molecular equipment allow us to ask again, more searchingly, how chromosomes and organisms, heredity and evolution, are connected and how they are separated in life; what, in short, they mean for one another.

Before the nucleus

Viruses and bacteria are not the precise ancestors of our nuclear and cellular organisms but they show us how such ancestors must have worked in several important respects. In the first place, they show us what must be the chemical basis of both heredity and development in all living things. It lies in the interaction of the two contrasted forms of nucleic acid, DNA and RNA. Evidently RNA arises from DNA in development, thereby creating the organism. But it can exist independently of DNA in the simplest viruses. Therefore in evolution it seems possible that DNA arose from RNA².

Second, bacteria have on a smaller scale the same linear arrangement of the nucleotides of DNA as do nuclear organisms. They have pre-nuclear chromosomes. They therefore mutate by the same rearrangements of linear sequences: substitution, duplication or loss, inversion or interchange. They recombine also by breakage and reunion of pieces of

chromosomes. But they have a circular form which does not allow either the diploid state, or meiosis or crossing over as known in nuclear organisms. On the contrary their rearrangements arise from processes of conjugation, insertion and infection which are elaborately controlled by the chromosomes, by subordinate bodies, plasmids, episomes and phages, and beyond them by enzymes generated by precise nucleotide sequences or genes³.

The results of this recombination in bacteria are less regular than in nuclear organisms and probably even more wasteful. They are achieved with a loss of inviable types, some redundancy and editing of nucleotide sequences, and overlap in activities such as are believed to occur at the higher stages of evolution⁴.

Our knowledge is defective in these respects but it already reveals a third principle of supreme importance, namely that the chromosomes of bacteria are organised for two adaptive functions: they regulate the metabolism of the bacterium, that is of the organism; they also regulate their own future modes of change, that is the evolution of their heredity. This is the same duality that we already know in the post-nuclear world.

To come to a fourth connection: we cannot be sure how far the transposable elements of nuclear organisms⁵ are vestiges of the episomes of bacteria; but we cannot doubt that the circular DNA of bacterial chromosomes corresponds to that of the organelles, the mitochondria^{6,7} and plastids,⁸ found in later evolution. In the origin of nuclear organisms, it thus seems that the ancestors of the nucleus and of the organelles have entered into symbiosis by infection. As I have argued, infection has become heredity⁹. If this is so, it follows that two stages of chromosome evolution now co-exist in the cells of all nuclear organisms. They also collaborate by rules that are not yet understood. It also follows that this origin has been telescoped with all the other successive and decisive steps in nuclear history, namely the invention of mitosis, meiosis and the alternation of haploid and diploid sexual generations.

Finally, the sequences of nucleotides forming units of activity in the bacterial chromosomes are the first in a hierarchy of other units of activity. For they determine the structure of transfer and messenger RNAs which in turn determine the arrangements of

amino acids in the structures of proteins¹⁰. Some of these proteins are immensely durable in evolution. The foundations of chlorophyll, cytochrome C and haemoglobin¹¹, had evidently been laid in the pre-nuclear stage and their genealogy can be traced through the great groups of plants and animals whose development they underwrote. Bacteria already had the machine tools with which the advanced industry of the cell was to be furnished¹².

Nucleus and chromosomes

The chromosome we were for long asked to take as our model was a cylindrical rod seen in fixed and stained cells at one stage of mitosis. Its motionless uniformity utterly contradicted the present and past functions that it was claimed to possess. Observation and experiment have gradually removed this contradiction. The bodies so neatly packaged at metaphase, conceal structures and activities and levels of organisation which we can now usefully survey.

The first example of structure and activity being connected was the 'spindle attachment' or centromere, a body responsible for the movements of each chromosome in cell division. Its functions were too big, too vital or too lethal, to be recognised in a breeding experiment. Yet it turns out to be a gene, one with a repetitive nucleotide structure such that, when broken by mis-division, its functions remain in its parts. The same is true of the nucleolar organiser which assembles RNA and proteins in the resting nucleus¹³.

These two structures and activities are immediately necessary for the survival, the one of the chromosome, the other of the nucleus. But both appear in the metaphase chromosome modestly disguised as constrictions. More completely disguised is the differentiation seen in the prophase of meiosis, in the lambrush chromosomes and in the polytene gland cells of flies. In those cells, the chromomeres, beads on a string, corresponding with the linear arrangement of genes, can be shown to have specific activities in protein production¹⁴. These activities vary between individual organisms and in the course of development. But they leave no trace in the metaphase uniformity of the living or conventionally fixed chromosome. To get inside the metaphase chromosome we have to use special methods and we then find a special kind of differentiation, not in beads but in blocks or segments.

The first step in tracing a differentiation of chromosomes in blocks was made by Heitz when he claimed that the property of failure to uncoil in the resting nucleus was specific and unconditional in certain segments of chromosome in plants and animals generally. To these segments which he argued were inert he gave the name of heterochromatin in contrast to the bulk of active euchromatin¹⁵. The second step was to make this resting stage differentiation visible at metaphase. In species of the wood-lily *Trillium* a period of freezing led to the appearance, at metaphase and anaphase of mitosis, of constricted segments. These segments were said to be starved of nucleic acid although the starvation, it now appears, was not of DNA but of the protein and RNA attached to it. Similarly at mitosis and meiosis in newts of the genus *Triton*. Soon many genera of plants revealed their starved segments and in doing so showed variation on a scale not previously suspected. Often there was so much variation in the species and consequent heterozygosity in the individual that every plant examined could be distinguished by its own chromosome pattern or map¹⁶.

The effect of starvation was to impair or modify the activity of H-segments (as they became known) in their three main mitotic aspects, reproduction in the resting stage, spiralsation in prophase and division or splitting in anaphase^{17,18}. These associated effects were attributed to *allocycly*: the synthesis of heterochromatin DNA at a different phase of the mitotic cycle from that of euchromatin DNA¹⁹. This supposition was later confirmed by autoradiography²⁰. Later an unspiralsed H-segment was revealed in the X chromosome of a mammal, the male hamster *Cricetus aureus*, by true starvation²¹. Also H-segments

in the bean *Vicia* were shown to be specifically broken by a particular reagent, maleic hydrazide, which presumably interferes with their reproduction^{22,23}.

A further step in exposing the block-differentiation of chromosomes was made when Caspersson in 1968, again using *Trillium*, found that an acridine derivative, quinacrine, would mark H-segments by differential fluorescence, either enhanced with AT bias or reduced with GC bias²⁴. Temperature treatment could now be bypassed and the way was open to use digestion and denaturation before staining as a means of distinguishing the proteins organised by particular H-segments in the metaphase chromosomes. At last in warm-blooded mammals and man, as we had seen long before in plants, individual chromosomes could be recognised and their genetic history traced²⁵.

These various methods revealed evolutionary contrasts. The first was between the whole complements of species. Within the best known plant genera *Allium*, *Trillium*, and *Fritillaria*, for example, heterochromatin may vary in amount from a trace to a third of the whole complement. Such a wide range suggests a directional trend in evolution in which the chromosome has cut loose from the needs of the organism or its environment^{26,27}. The second is between individual chromosomes. For some chromosomes (even X and Y in primates) prove to be unstable within a species or a race while others are stable over whole orders and classes²⁸.

These discoveries answer some evolutionary questions. But they raise new ones. How far does block or banded differentiation correspond with gene or chromomere structure? Clearly they represent the different needs of chromosome organisation at different stages in the cycle of the cell and of the organism²⁹⁻³¹. How different these needs are is shown by the conditional behaviour of some heterochromatin. It may vary in relation to stage of development, position in relation to the centromere, haploidy or diploidy or even the action of an adjoining gene³². The most striking example of the last two appears in the X chromosomes of man and the mammals; notably in the mosaic coat pattern of the female tortoiseshell cat.

It seems that there is a hierarchy of control mediated in cellular organisms as in bacteria by enzymic messengers operating at many levels between the nucleotide and the whole cell and often canalised along chromosomes³³. This hierarchy will have to be separately deciphered in many groups of plants and animals. But it already tells us that there is a division of function and hence an opposition of interest in the chromosome between its own needs and those of the cell and the organism. Most significant in the long term are the effects of this division on behaviour at meiosis.

The evolution of the sexual cycle

A sexual cycle of alternate conjugation and meiosis is characteristic of every group of nuclear organisms. Ninety years ago (just when the word chromosome was coming into use) Weismann suggested that this alternation was itself responsible for evolution: it secured a recombination of hereditary differences which would expose the variation of organisms to natural selection³⁴. Two kinds of breeding experiment have in the intervening years turned Weismann's speculation into the main instrument of evolutionary thinking.

On the one hand experiments with organisms have shown that conjugation is the means of bringing differences together. And on the other hand experiments with chromosomes have shown that meiosis is the means of pulling them apart. Further both of the two complementary processes have proved to be under genetic control. But beneath this control a third variable is crucial: the time sequence of fertilisation and meiosis which we call the sexual cycle. These three variables are inevitably connected in evolution.

The sexual cycle evidently began in evolution with meiosis coming straight after conjugation as though no diploid mitosis were possible. This haploid supremacy is seen only in the

Protista and lower plants. From it animals seem to have moved abruptly into the opposite state of a complete diploidy; so abruptly that if they stood alone this revolution might have left us nonplussed. But a gamut of transitional stages is preserved in plants. In them meiosis is deferred by a series of steps, which have proved effectively irreversible. In this way the diploid phase has displaced the haploid, finally in a few Protista or Algae even extinguishing it (Fig. 1)¹.

In multicellular organisms, it has long been supposed that the diploid phase has expanded owing to the evolutionary advantage of its enlargement of the range of variation. The difference in its expansion between animals and plants we can now see is due to the different requirements of our second variable, the control of fertilisation, or the restraint of both outbreeding and inbreeding. In animals this control lies almost entirely in the mobile behaviour of diploid organisms. In plants it lies in the protein reactions governing incompatibility in their haploid and diploid phases. These reactions are now thought to have a

evolutionary experiments. All of them concern genetic recombination as we can tell when either of the components of sexual reproduction, conjugation or meiosis, fails.

Characteristically in parthenogenetic plants and animals the two processes are suppressed together³⁷. But without suppressing either, in hermaphrodite plants a mutation to self-fertility can quickly bring recombination to a standstill. In either case variation ceases. The stock is fixed as a stable race or species, adapted perhaps but no longer adaptable. So long as sexual reproduction continues without inbreeding, however, the option is left open and adaptability is preserved for the future.

Naturally, except in domestication³⁸, plants and animals as a whole have kept the option open. They are adapted to preserve the future interests of the species. These embrace the need to maintain, in changing environments and in successive generations of organisms, the cooperation of chromosomes and nuclei, mitochondria and plastids, genetic materials and agents, whose relations, as we saw, are relics of the past. When we recall that failure at any point may lead to sterility, a full stop, we understand why this problem of internal adaptation, of mutual selection, baffled Darwin and was avoided by the Darwinians.

The adaptation of meiosis

To understand the taking apart of the differences brought together in the sexual cycle we must take note of the inherent paradoxes of meiosis. For meiosis connects individuals and generations; it also separates them. At meiosis the DNA code is organising both an unlimited continuity of organisms and a repeated discontinuity between them. And in meiosis the chromosomes make themselves the agents of a drastic choice between stability and variation. How do they do it? By a variety of devices modifying meiosis which arise, to use Wilson's phrase, as natural experiments³⁹. For they appear in all animal and plant groups throughout evolution without regard to affinities or lineages or levels of organisation.

The most widespread of these devices springs from the earliest manifestation of sexual differentiation itself. In all kinds of organisms whether hermaphrodite or separate-sexed, in which small sperm fertilise large eggs, these germ cells are produced by way of two sizes of cell and hence by way of two forms of meiosis. In its common origin before sexual differentiation this meiosis must have uniformly required pairing, crossing over and chiasmata between parental chromosomes¹. But one of the two forms, male or female, later often found another way of doing it. Reduction, segregation and recombination were achieved without crossing over, or the formation of chiasmata, or the separation of genes within the chromosome. Thus in one sex, always the XY sex where there was one, never of course in both sexes, whole chromosomes were inherited as a unit⁴⁰.

In this way two tracks had been laid in heredity. On one track new chromosomes are constructed by crossing over in every generation. On the other, old chromosomes are kept intact potentially forever. This elegant device was the key to the controlled breeding of *Drosophila* in experiments. But for some time before it had been the key to the breeding of flies in nature and their evolution⁴¹.

The two-track system works in a variety of ways. Meiosis without chiasmata is the simplest. It is found not only in flies but also in mantids and scorpions, moths and mites as well as in certain hermaphrodite plants^{42,43}. Haploidy in the male sex is another way of reaching the same goal. It allows bees, wasps and ants, and single species of beetles and bugs, to avoid all recombination in the male sex¹³. But it has one drawback, that these males have no offspring of their own sex. Most widespread of two-track systems is probably that in hermaphrodite plants and in some Amphibia where the large chromosomes allow us to see the frequency and distribution of chiasmata sharply or slightly contrasted between the two sexes^{41,44}.

The mechanisms of two-track heredity are thus slight or drastic, direct or devious. In them heterochromatin and accessory chromosomes sometimes play a part whose working

The Cycles of Sexual Reproduction in Plants and Animals

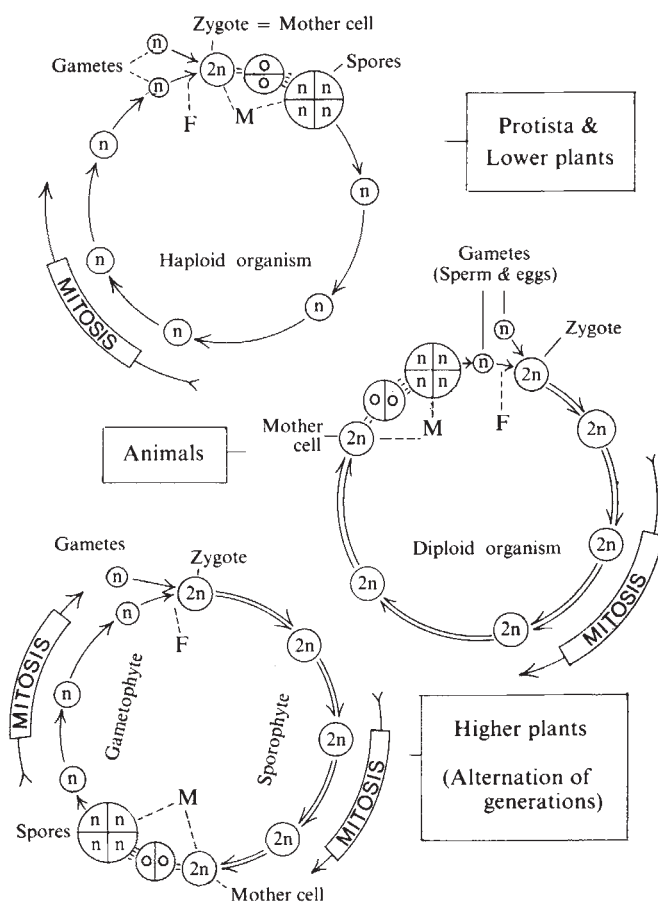


Fig. 1 Diagram showing the three types of sexual cycle found in nuclear organisms to illustrate their evolutionary connections. F, fertilisation or conjugation; M, meiosis; n and 2n, single and double lines, haploid and diploid.

genetic continuity all the way from their bacterial origins³⁵. In the Angiosperms the haploid phase is preserved because in addition it is manifestly indispensable for the mechanisms of the style and of double fertilisation which provide the most exact and versatile of all genetic controls of the breeding system and the one which was evidently responsible for the origin of the angiosperms³⁶.

Such is the macro-evolutionary panorama. Behind it however the breeding of organisms and of chromosomes now reveals an array of smaller events, the surviving successes of repeated

remains to be explored. But in its consequences (which are its evolutionary causes) two-track heredity is sharply definable. So long as outbreeding is maintained, the choice of track gives the sexual process an unforeseen, and hitherto unrecognised, scope and power. For not only does it generate diversity through the uncertainty it organises in recombination. It also organises its distribution selectively in succeeding generations.

On this basis we can look again at the evolution of sexual reproduction as a whole. Its origin and all its adaptations now show us that it concerns not the organisms in which they began but the selection and survival of their descendants. Never are they concerned with adaptation to any present environment; always either to future environments or to future internal arrangements in the species, sometimes in long hierarchical successions as in, longest of all, the evolution of the life cycle itself.

What has made this possible is the continuity of the chromosomes from generation to generation. Their continuity (or immortality, to use Weismann's term) overrides the discontinuity or individuality of organisms which has over-awed our naturalists: embryologists and paleontologists, Darwinians and Mendelians alike, have taken it as the axiom of axioms. But it is the continuity of the chromosomes which has allowed genetic systems to be built on natural experiments; these experiments have been tested on individuals, but they have been maintained by communities which like the chromosomes are extended in time and space beyond the individual, beyond the organism.

Direction and feedback in selection

Comparisons of organisms in both their living and fossil anatomy have long convinced us that they often show great trends in the evolution of their bodily structure. These trends were first given the name of *orthogenesis* by paleontologists who saw a convenient Lamarckian interpretation for them⁴⁵. They are, however, easily understood as the product of positive feedback in natural selection where one change favours rather than disfavors another in the same direction. If this is so we have to ask whether such trends occur at the other levels of evolutionary change which we now recognise, and with what effect.

In fact we find trends at all our lower levels of evolutionary change. Examples are: repetitive sequences in DNA^{46,47}; extreme proliferations of heterochromatin⁴⁸; multiplication of chromosomes by polyploidy in plants³⁸; by fragmentation in both plants and animals (giving bimodality of chromosome size in many groups)^{38,43}; multiplication of accessory and X chromosomes⁴⁹⁻⁵²; decay of the Y chromosomes^{39,49}; expansion of interchange hybridity giving ring formation at meiosis^{38,40}; and extravagant incompatibility allelism in plants³⁵.

With this variety of examples we can now ask another question, whether any connection exists between trends at different levels. The answer is that no such connection seems to exist. A change at one level may no doubt be responsible for initiating a trend at another level, but no more.

Take the polyploidy that is seen in flowering plants. It sometimes leads to a series of multiples: 4, 8, 16, 32 and so on. The scale and range of this occurrence in thousands of genera, is now so well known as to constitute a vast natural experiment³⁸. Now polyploid plants arising in cultivation are enlarged in size and plant breeders welcome this gigantism. But in nature the plants themselves do not. On the contrary they either reject it or correct it. Even, we may say, when they reduce their chromosome size, they actively disconnect it from their bodily structure¹³.

What then do the polyploid trends achieve?

Evidently each jump has separated a new stock from an old one. By inter-sterility it has initiated a new species isolated genetically from its parent⁵³. The same is true in less degree of all changes of chromosome number in both plants and animals. But it is also true that these changes are commoner where the

organism is small and short-lived so that the species can afford to experiment; they are less common where the organism is large and its slow propagation makes the gamble more costly. Notably is this so in the flowering plants and in our own group, the primates⁵⁴.

Interchanges between chromosomes demonstrate the same principle. How do they establish themselves? In all outbreeding plants and animals migrating colonies are liable to be isolated and so compelled to inbreed. In this emergency parthenogenesis is one means of preserving the reservoir of variation and hybridity they may need for survival. Polyploidy is another. A third is interchange^{38,55}. Two chromosomes AB and CD exchange segments to give BC and DA. The nucleus is then hybrid and the chromosomes, old and new, can no longer simply pair. At meiosis they form a ring of four: AB-BC-CD-DA. Such a ring can give viable gametes only of two types, the old and the new, AB, CD and BC, DA. Either of these uniting with a similar partner will give a complete homozygote with impaired viability. But by uniting with the other type it will keep the full heterozygosity of its parent. The interchange hybrid with its ring of four therefore both grows well and breeds true. Further interchanges can enlarge the ring to six, eight and on to fourteen. Each step in this trend feeds back to make another step in the same direction more desirable. Finally all the variation available in the outbred community will be trapped and held in one inbred and now true-breeding hybrid stock or species.

Thus the effect of interchange is to raise barriers to recombination not through crossing but through crossing over, not at the diploid but at the haploid level, and not between organisms but between chromosomes.

The classical instances of this process are the species of *Oenothera* which have been forced into their evolutionary trend by a conversion from outbreeding to inbreeding, itself promoted by migration across North America after the retreat of the ice. De Vries founded his mutation theory on the breeding behaviour of these species and we can now at last do justice to his much-disputed argument⁵⁶. The building up of his hybrid species clearly depended on connecting the two primary initiatives, of genic self-compatibility and chromosome interchange. Both of these were internal or genetic changes. Both we may call mutations. But it was through the breaking down of these hybrid species, their secondary evolution through hybridisation in Europe, that de Vries came on what he called mutations¹³.

Oenothera thus shows us both the building up and the breaking down of species. And in both processes we are left agreeing with de Vries that the selection of organisms by adaptation to the environment is secondary to the internal, lower level, processes of mutation. At the same time we discover that the trend in building up interchanges has no connection with any trend in the bodily evolution of the organisms, the plants³⁸.

Most notable for their evolutionary trends are the sex chromosomes in their XX-XY systems of determination. Both X and Y have two parts, a pairing segment in which the two exchange by crossing over, and a differential segment without crossing over and hence, for the Y, confined to one sex⁴⁰. This restriction as we might expect makes the Y especially vulnerable and its evolutionary disappearance has been independently inferred in most large animal groups^{42,43}. Organisms in which the loss has occurred do not however exploit it. On the contrary we again have to say that they correct it. They accept interchange of X or Y with a pair of autosomes. In doing so they often initiate a trend. They build up a ring or chain of chromosomes at meiosis in the heterozygous sex. This process reaches a climax in a number of plant and animals: 3 chromosomes joined in a marsupial⁵⁷ 5 chromosomes in a hop¹³ 8 in a mistletoe⁵⁸ 9 in a centipede,⁵⁹ 14 in a termite,⁶⁰ 9 or 10 in each of those remarkable relics, the monotreme mammals⁶¹.

In these examples the heterozygous sex extends its fixed hybridity while allowing the homozygous sex to continue its free recombination. Two advantages have thus been combined and reconciled in one system: the true-breeding hybridity of *Oenothera* and the two-track heredity of *Drosophila*.

If we look at instability in general at different levels this conclusion is strengthened. Evolutionary similarities of many kinds can now be compared. Organisms provide us with our conventional and systematic standards. The chromosomes show a variety of evolutionary trends. Proteins such as isozymes⁶² or haemoglobins⁶³, and sequences of DNA show much greater stability. But if we look at each group separately we find that each has its own character. As between man and the apes it is the brain that has changed most, the blood and the DNA least, and the chromosomes lie between⁴⁷.

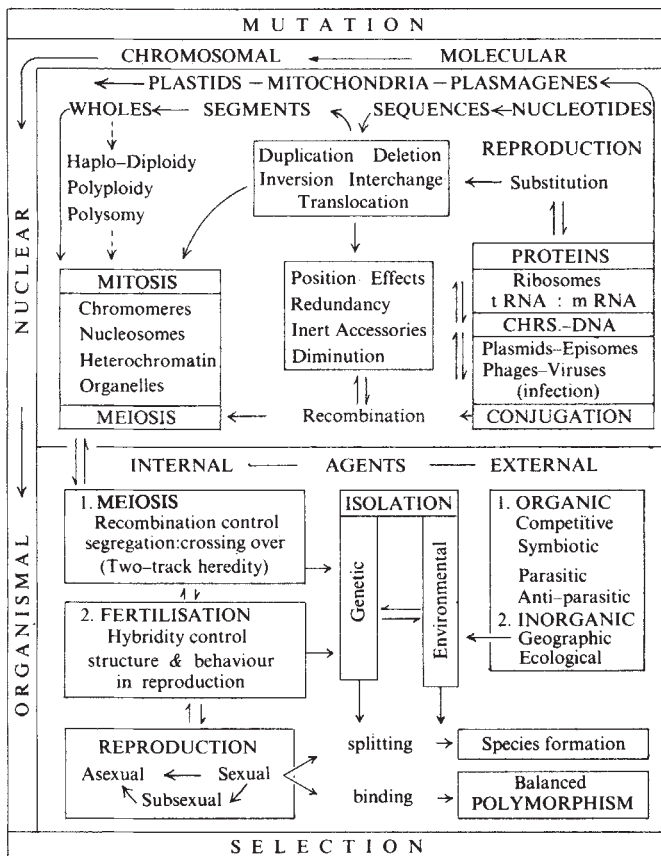


Fig. 2 Diagram showing the multilateral evolutionary relations of molecules, chromosomes, organisms and species subject to mutation at lower levels and selection at all levels.

Although DNA, genes and chromosomes, organisms and species are connected in activity they are separated in selection. It is as though they were protected from one another in stability. We do not know how this happens. But it is utterly clear that if trends at the molecular and chromosome levels are demonstrating feedback in selection so also are trends in the giraffe's neck or the vertebrate's brain. And the Darwinians after all need not have been embarrassed by the notion of orthogenesis^{45,64}.

The origin of species

The evolution theory began with organisms. For Darwin (in the first and in the last edition of the *Origin*) natural selection worked 'solely by and for the good of each being', that is of each organism⁶⁵. And, if it worked on heredity, that heredity was a property of the organism. In the same vein Morgan wrote that 'the chromosomes fulfill the requirements of genetics' meaning that groups of genes do what organisms require them to do^{66,67}. Which is true if we allow that the experimenter is seeing nature back to front.

Again when Darwin wrote that species 'originated' as varieties he meant that they arose from differences between organisms.

Haldane therefore, putting Morgan's genes into Darwin's organisms, might claim that differences between varieties were 'generally due to a few genes with relatively large effects' while the chromosomes were sometimes convenient in assembling the genes to make still larger differences between species. In principle the mutation of genes, the spatial isolation of organisms, and the natural selection of one through the other, made varieties into species. If the details might be left in the dark, these were the 'causes of evolution'⁶⁴ (see Fig. 2).

At this point, however, a number of details were being brought into the light. Differences between related species sometimes seem to have no adaptive connection with their external environments. Might not sampling errors in mendelian assortment be responsible? In small populations, Sewall Wright suggested, these errors would check the smooth working of natural selection^{68,69}. Slowly we have come to see the scope of this principle. But the sampling we notice concerns not just the organisms and genes of Wright or Haldane but the whole gamut of variation from the nucleotide to the species itself. It is indeed a hierarchy with many interactions which we need to examine.

Every new polyploid begins as the smallest of all populations, a unique individual. And it is cut off from its parental diploid stock, often for ever. It is therefore at once a potential new species. In a different way every new inversion within a chromosome is a unique individual. As a heterozygote it is prevented from crossing over with its sister segment, often for ever. Every new interchange likewise yields a unique individual. All of these establish, by an act of sampling, new populations of chromosomes, and nuclei and organisms. And even when the changed chromosomes lie together with their alternatives in the same cells they are genetically isolated from one another^{53,70}. For, in their possibilities of recombination, they might as well be ages or oceans apart.

Here *Oenothera* illustrates both the rule and its exceptions. For, in the hybrid species, the genetic isolation of gametic alternatives is not always for ever. It may break down, by crossing over. It then gives mutations; but, unlike the new mutations of *Drosophila*, they are not always deleterious since they are due to combinations of chromosomes already tried out by selection.

We can now see that all outbreeding species are fields of genetic recombination; that any blocking of recombination splits the species; and its source may be, indeed must be, either external or internal, either environmental or genetic. Further we can sometimes see which it is; but not always, since either may quickly promote the other. We can however see from our experiences of chromosomes that when the initiative is external the populations separated will contain more genetic differences than the naturalist would imagine. But not all such differences will contribute to the splitting of a species. Throughout plants and animals the variation within a species is partly concerned with the opposite task, the long-continuing task of holding the species together.

This principle is true at all our levels of analysis and it is best described in terms of the balanced polymorphisms of Ford. Blood groups, immunity reactions, and mimicry systems in animals and the incompatibility alleles of plants all serve to bind a species together⁷¹. Our knowledge of heterochromatin bridges the gap between these systems and the sex chromosomes of plants and animals. In the heterozygous sex these in turn may be linked with inversions as well as interchanges. An example is the 'sex-ratio' super-gene in *Drosophila*. The males that carry this floating recessive complex correct the sex-ratio of the population by having an abnormal meiosis which gives largely female progeny. The complex is based on inversions, three of them overlapping, and it is common to a group of many species. It is not as old as the X chromosome but it is clearly older than all the species together that carry it⁷².

Consider now the entire range of variation arising in a species at all material levels from the nucleotide to the nucleus. It is evidently available to the species for two kinds of selection and adaptation. These are variously developed and compete with

one another. On the one hand, older variations are largely pre-empted as polymorphisms which serve to bind the species together. On the other hand, newer variations are always becoming available which may serve to split it apart. But when this split succeeds it still largely fails to break the continuity of the older polymorphisms.

The competition between these choices means that we have to explore the balance between the present needs of the individual and the future needs of the species, between the internal and the external opportunities of each community. Sometimes as with the origin of the Angiosperms the incompatibility super-gene may have both splitting and binding functions open to it⁷³. And sometimes, as in the beginning of man, a mutational initiative in a single cell, by the union of two chromosomes to make one, may decide the origin of a new species or new family⁵⁴. In Fisher's hands natural selection might have seemed to be a truism⁷⁴ but these choices and conflicts that are now open to our examination have nothing of the truism about them.

The use of a diagram

Darwinians have seen evolution as the product of mutation and selection. We may agree. But for them mutation, the primary process, acted at the lowest level in their scheme, which came to be known as the 'gene'. And selection, the secondary process, acted, at the highest level in this scheme, that of the organism. For us the physical continuity exists, and the mutation and selection therefore act, at many levels. For us mutation runs all the way from the single nucleotide to the nucleus and the plasmagene. And selection runs all the way from the tri-nucleotide to the community of organisms and, what is more, through a succession of generations. Selection is therefore enormously extended in time and in space.

To use this knowledge we need to take to pieces and reassemble both the assumptions and the conclusions that our predecessors, properly in their time, attempted to establish. We need to think of the relations between levels and systems and in order to do so we need a new framework, a new diagram. Even in its two dimensions such a diagram may help us to see both the connections and the gaps in our understanding.

What we see is heredity changing and using its materials and its processes, derived from the past, to meet the needs of the

present and the hazards of the future. It does not show us how often, as in human affairs, the long-term advantage is set aside in favour of short-term opportunity. But it allows us to see how very long the past is both for molecules and for chromosomes. And above all it allows us to see that behind the mendelian choice between change and no-change lie a succession of deeper alternatives within heredity itself: between control by the nucleus and by the cytoplasm; between the fixing or recombining of nucleotides, of genes, of segments and of chromosomes; and between the binding or splitting of sexes, of races and of species.

In all these choices mutations are the basis of future adaptations and evolution. They are pre-adaptations not just one stage but a lengthening succession of stages in advance of the transformations that overtake organisms and communities. Each of the great steps in this process, beginning with RNA and DNA, L-amino acids and proteins, has in turn prescribed some future developments and proscribed others. All have curtailed darwinian randomness. Among the greatest steps of all, some were telescoped in the invention of the nucleus and the nuclear chromosome, mitosis and meiosis; others were fully exposed in the evolving sexual cycles.

When, in 1871, Matthew Arnold suggested that Darwin's ideas were 'all in Lucretius' in one respect he understated the case⁷⁵. To atomists, old and new, changes of atoms were bound from the beginning to have that priority over selection of which Darwin was uncertain^{76,77}. And it was only in the last stages of evolution, and only to us today, that the consequences of this priority became unmistakably clear. For the little changes which came near the end gave that genetic control of fertilisation, and through gestation of development, to which first the Angiosperms and then the mammals owed their origins^{36,37}.

In these processes the forward-looking or experimental capacity of selection culminated. For they allowed heredity to control the environment not only of each organism but also of the following generation. What was in neither Lucretius nor Darwin was the extension of this principle which has been demonstrated by the history of man himself, the extension which allowed individuals to create the environments of indefinitely succeeding generations. Whence sprang a process of directed evolution with a feedback so formidable that it is only now brought within reach of our enquiry⁵⁴.

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articles

Eternity is unstable

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We show that if the Universe expands forever, it will become increasingly irregular in the far future. In particular, we demonstrate that the Hawking black hole evaporation process causes a vortical instability to develop in spatially homogeneous spacetimes which are approximately $k=0$ Friedmann universes. We associate the increase of irregularity with the increase of gravitational field entropy, and thus provide a novel picture of the universal heat death.

THE aim of this article is to describe, in the light of recent developments in gravitation physics, the likely future state of the Universe if it expands forever. We shall be especially concerned with extending the analysis of Collins and Hawking (CH), who in their classic paper¹ studied the asymptotic stability of the ever-expanding Friedmann universes under the action of generic, homogeneous perturbations. Their aim was to test the plausibility of Misner's idea that our Universe, now virtually isotropic, could have arisen from an arbitrarily non-uniform set of initial conditions^{2,3}; its present regularity being guaranteed solely by the laws of physics with no memory of initial conditions. They examined the most general spectrum of homogeneous perturbations to the flat, ($k=0$), and open ($k=-1$), Friedmann models, and concluded that unless the Universe were neighbouring the special state of zero total energy ($k=0$), the property of isotropy is unstable. Only initial data of zero measure in the metaspaces of all possible cosmological Cauchy data give rise to universes of zero total energy which are able to isotropize in the future. In general, the open universes with non-zero total energy will become increasingly anisotropic with the passage of time^{4,5}. The Collins–Hawking analysis tells us that either the Universe is young—instabilities having had insufficient time to grow remarkably as yet—or that the Universe is in a highly preferred, zero measure, binding-energy state. They opt for the second alternative by invocation of the anthropic cosmological principle^{6–8}, which we discuss below.

By extension of the CH theory we shall show that there are novel consequences for the future dynamics and thermodynamics introduced by particle evaporation from black holes^{9,10}. It transpires that the non-conservation of baryon number of the gravitational interaction¹¹ creates an inevitable radiation-filled environment in the far future of a universe which approximates the zero energy Friedmann model. This thermodynamic state renders such universes unstable to the development of vorticity and random peculiar velocities. These universes can no longer isotropize in the manner defined by Collins and Hawking; they become increasingly dominated by the centrifugal and kinetic energy of material motions rather than the particle rest energies and tend towards a dynamical state that may be viewed as radiation-dominated, relativistic vortex.

Thus, the Collins–Hawking proof of the instability of the $k=-1$ Friedmann universe coupled with our proof of the instability of the $k=0$ Friedmann universe shows that all ever-expanding universes are unstable: they do not isotropize at large times.

In the following sections, we first give a short history of the studies dealing with the final state, and then we describe the possible and probable outlines for the classical and quantum evolution of the Universe into the far future. The consequences of this discussion for the stability of Friedmann universes are calculated and some further general ideas regarding the asymptotic behaviour of the Universe ventured in the final section.

The past

Eschatological studies of the Universe have periodically attracted the interest of many physicists, philosophers and astronomers^{12,13}. These investigations have, in the main, been precipitated by the second law of thermodynamics which arose as a generalisation of Carnot's work¹⁴ in 1824 on the efficiency of heat engines. In 1852 Lord Kelvin extended Carnot's work and propounded¹⁵ the "universal tendency in nature to the dissipation of mechanical energy". Two years later, Helmholtz made the first explicit statement of the now familiar notion of the 'heat death of Universe'. He envisioned an ultimately stable state of all-encompassing thermodynamic equilibrium¹⁶: "the Universe from that time forward would be condemned to a state of eternal rest". Clausius echoed Helmholtz' ideas as follows^{17,18}

"the more the Universe approaches the limiting condition in which the entropy is a maximum, the more do the occasions of further changes diminish; and supposing this condition to be at last completely obtained, no further change could evermore take place, and the Universe would be in a state of unchanging death."

These ideas were widely discussed in the latter half of the nineteenth century and indeed had a profound influence on the *Weltanschauung* of that period^{19,20}. Jeans²¹ and Eddington²² were responsible for bringing the notion of the 'heat death' into relativistic cosmology. Eddington asserted in 1931 that²³

"It used to be thought that in the end all the matter of the Universe would collect into one rather dense ball at uniform temperature; but the doctrine of spherical space, and more especially the recent results as to the expansion of the Universe, have changed that... It is widely thought that matter slowly changes into radiation. If so, it would seem that the Universe will ultimately become a ball of radiation growing ever larger, the radiation becoming thinner and passing into longer and longer wavelengths. About every 1,500 million years it will double its radius, and its size will go on expanding in this way in geometrical progression for ever."

Eddington's view of the Heat Death has important similarities with the picture of the Heat Death derived in what follows.

The classical future

Let us first consider the ultimate fate of the large aggregates of matter that populate the present day Universe. As time passes, clusters, galaxies and other stellar systems will become increasingly bound by gravitational forces since they will radiate away their binding energy in the form of gravitational waves. Dead stars with mass exceeding the Landau–Chandrasekhar limit, $M_{LC} \sim \hbar^{3/2} m_n^{-2} \sim 1.5 M_\odot$ will be the first objects to become black holes, but galaxies and the largest bound configurations $\sim 10^{17} M_\odot$ will also eventually follow them. The time taken for the radiation of the binding energy to precipitate clusters into black holes may be roughly determined from the time scale on which these structures will change their quadrupole moments. Equating the gravitational energy²⁴ $\sim M^2 R^{-1}$ with the rotational energy we obtain from the Landau–Lifshitz radiation formula^{25,26} a lifetime $t_g \sim G^{-3} c^5 R^4 M^{-3}$ seconds against gravitational wave decay.

The time scale for a large $10^{15} M_\odot$ cluster to evolve into a black hole state under the assumptions of these naive arguments is $\sim 10^{19}$ yr.

This radiative loss of binding energy is the principal means whereby the largest aggregates of matter will become increasingly bound into trapped, black hole states. Another is by the ‘evaporation’ from their interiors of objects which gain sufficient kinetic energy from gravitational deflections, encounters and instabilities to attain escape velocity. Such ‘evaporation’ will clearly accelerate the central core’s collapse to a compact state since objects with positive energy will be diffusively ejected from the system leaving its total energy thus more negative. The details of such mechanisms are exceedingly complex because of subtle collective relaxation effects²⁷, but the time scale will clearly be related to the relaxation time for gravitational encounters^{27–30}.

Clusters will for example evaporate galaxy-sized objects in $\sim 10^{11}$ yr and stellar-size ones in $\sim 10^{23}$ yr. Of course, many of the assumptions used to generate these numbers have been stretched to the limit (and beyond) in this context; it serves merely to illustrate that evaporative effects will occur to augment the radiative decay of self-gravitating systems.

Material may be cycled through infall and evaporation many times as the Universe moves inexorably towards a state consisting of supermassive black holes, debris in the form of ‘dead’ matter, elementary particles, and radiation. The fate of these components will ultimately be determined by quantum processes and we now turn to consider them.

The quantum future

The preceding section traced the evolution of an ever-expanding universe until its matter resided in essentially four basic states: (1) black holes; (2) condensed objects such as neutron stars, black dwarfs, and various clumps of matter; (3) dispersed elementary particles; (4) radiation. However, the evolution of the matter will not stop here. In fact, we wish to argue that material in the first two states will eventually be converted via quantum processes into the third and the fourth types, and that those open universes now neighbouring a $k=0$ Friedmann universe will eventually become radiation dominated.

Condensed objects of type (2) will be converted into either black holes or dispersed elementary particles by quantum tunneling processes. The zero-point motions of the particles comprising a neutron star or black dwarf could cause the surface of the star to fluctuate inside its Schwarzschild radius³¹, or else impart escape velocity to a particle on the stellar surface. If the former does not occur, then the latter process will eventually lead to the complete evaporation of the star: its particles will become completely dispersed³². As an example of this dispersal process, consider the decay of a heavy nucleus. Its constituent particles can tunnel through the potential barrier as long as the total mass-energy of the escaping particles is balanced by an increase of nuclear binding-energy, and this will be the case until the nucleus has decayed into ^{56}Fe . As no nucleus has a greater

binding energy than ^{56}Fe , further decay can proceed only after nuclear baryons decay, and the decay will be caused by the Hawking process discussed below. The decay of a black dwarf is similar. It will first decay into an iron star, then a neutron star, and then either tunnel into a black hole, or evaporate as its constituent baryons decay.

Hawking has shown¹⁰ that a black hole will emit black body radiation at a temperature which is inversely proportional to its mass. A black hole thus cannot be a final state of matter. Instead, most of a black hole’s mass will eventually be turned into radiation, and a small fraction into high-velocity elementary particles. (Copious particle production will not occur until the final stages of the black hole evaporation when the black hole temperature reaches 10^8 degrees, see ref. 33). A black hole will not begin to radiate away its mass until the expansion of the Universe cools the ambient radiation below the black hole surface temperature, but in an ever-expanding Universe, this will inevitably happen. To see this inevitability, note that there are two effects which act to augment the mass of a black hole: the accretion of matter and radiation by a black hole, and the coalescence of black holes into larger ones. The complete evaporation of black holes will occur if the ambient radiation temperature, which is proportional to $1/R$, is in the long run being reduced faster than the two above mentioned effects can reduce the temperature of black holes, which is proportional to $1/M$. Carr³⁴ has shown that for black holes which are initially smaller than the particle horizon, accretion by matter and radiation in an environment where the average matter density is proportional to the cosmological density cannot increase the black hole mass by more than an order of magnitude in the entire future history of the Universe. At late times, the matter density near a black hole should be proportional to the cosmological density. Furthermore, no black hole whose event horizon does not intersect the initial singularity (that is, non-*ab initio* black holes) can be larger than the particle horizon (this fact can be seen by inspection of the appropriate Penrose diagram). Thus if we make the physically reasonable assumption that there are no *ab initio* black holes, we have a cutoff at high mass in the black hole mass distribution at any given time; the Carr argument then tells us that there is an upper bound to the mass of a black hole, and hence a non-zero lower bound to the black hole temperature, at all times. This assumes, of course, that the black holes cannot coalesce to form black holes of arbitrarily large mass. To calculate the increase of black hole mass due to coalescence, note that the rate of increase of mass due to black hole collisions is proportional to $\sigma_{\text{BH}} \rho_{\text{BH}} \langle v \rangle$, where σ_{BH} is the black hole capture cross-section (this factor includes the potential of the gravitational Coulomb effect to capture other black holes). ρ_{BH} is the black hole mass density, and $\langle v \rangle$ is the average velocity of the black holes with respect to the fundamental observers. Novikov and Thorne³⁵ have given $\sigma_{\text{BH}} \propto M^2 \langle v \rangle^{-2}$, and so we have $dM/dt = \rho_{\text{BH}} M^2 \langle v \rangle^{-1}$. Now $\rho_{\text{BH}} \propto 1/R^3 = 1/t^2$. Assuming $\langle v \rangle \propto t^{-m}$ and integrating, we see that M is bounded above even in the limit as $t \rightarrow +\infty$ if $m < 1$, which will hold if $\langle v \rangle \propto 1/R$ or $1/R^{1/2}$. This means that the total number of collisions occurring in infinite time will be finite in the Friedmann universes considered. Thus in any ever-expanding Universe, black holes will eventually completely evaporate; in the far future of an open Universe, all matter will be reduced to elementary particles and radiation.

But the evolution of matter will not cease here. A careful consideration of the Hawking process leads to the conclusion that protons and electrons—supposedly stable elementary particles—are in fact unstable and will decay into massless particles. The ‘no-hair’ theorem²⁴ tells us that a black hole is characterised by only three parameters: mass, angular momentum, and electric charge. Hence, there is no way to distinguish between a black hole formed by the collapse of matter from a black hole formed by the collapse of anti-matter. The Hawking process causes an equal number of particles and anti-particles to evaporate from a black hole, so if a black hole which completely evaporates was originally formed of matter, the combined

process of black hole formation and evaporation leads to a net decrease in the total baryon and lepton number in the universe. That is, the Hawking process can violate the conservation of baryon and lepton number. Since general relativity contains no intrinsic length scale, black holes of any size can in principle exist; this tells us that the gravitational interaction intrinsically violates lepton and baryon number. (One way of thinking of this is $p + e \rightarrow \text{BH} \rightarrow 2\gamma$). That is, the proton and electron comprising a hydrogen atom can quantum-mechanically tunnel inside their combined Schwarzschild radius to form a black hole, which then decays via the Hawking process into two photons). Some decay modes of electrons and protons via this gravitational interaction are



Protons and electrons are known to be stable within the limits of experimental measurement³⁶, so the time scale for the gravitational annihilation of baryons and leptons must be extremely long—a rough calculation by Zel'dovich^{37,38} gives a half life t_0 of 10^{45} yr. This half life is very small when compared with eternity, however, and all baryons will eventually decay into leptons and radiation. The charged leptons themselves do not decay because the gravitational interaction conserves electric charge.

The baryon mass density will be proportional to $e^{-1/t_0 t^{-N}}$ at late times, (at least until the reverse reactions of (1) become important. In the low temperature regime considered here, the reverse reactions will be dominated by the zero point fluctuations considered below) where the value of N depends on the effective equation of state ($N = 2$ and $3/2$ for matter-dominated and radiation-dominated cosmologies respectively). The ambient radiation density is proportional to t^{-N} , so the radiation density will eventually dominate the baryon density. Neglecting for the moment the zero-point fluctuation, the material of the Universe at late times will essentially consist of an electron-positron plasma in a radiation bath. One might expect that the electron-positron pairs would rapidly annihilate leaving only radiation, but in an expanding universe, this does not necessarily hold. For a sufficiently rapid expansion, the electron-positron pairs may become the dominant form of matter. The mass density of the electron-positron pairs in a Friedmann universe is given by

$$\frac{d\rho}{dt} = \frac{-3\rho}{R} \frac{dR}{dt} - \sigma_{\text{Thomson}} \langle v \rangle \rho^2 - [\text{electrical attraction term}]$$

For all ever-expanding universes, the second term becomes much smaller than the other terms at very late times, (contrast the black hole coalescence effect discussed earlier). For $k = -1$ universes, or universes which approach this class of Friedmann universes in the infinite future limit, the first term dominates and the electron-positron mass density controls the expansion. However, for ever-expanding universes neighbouring a $k = 0$ Friedmann universe, the electrical attraction term will cause the electron-positron mass density to decrease faster than the ambient radiation mass density, so that eventually the former can be neglected in comparison with the latter.

The physical mechanism for this is as follows. In $k = 0$ Friedmann universes, the fundamental observers have zero gravitational binding energy. This means that electron-positron pairs will become electrically bound—and hence eventually annihilate—if $\frac{1}{2}m\langle v^2 \rangle$ becomes smaller than the electrical potential energy of a pair (which is proportional to e^2/R), where $\langle v^2 \rangle$ is the mean square velocity of an electron or positron with respect to the fundamental observers. Now $\langle v^2 \rangle \propto 1/R^2$, and so the electrical potential energy of a pair will eventually become larger than the kinetic energy—an electron-positron pair will become bound. The time needed to annihilate will depend on the initial distance at which the particles first become bound, and so if the initial spectrum of velocities is either maxwellian or determined by the decay of protons via the Hawking process (the number

density of protons being proportional to $e^{-1/t_0 t^{-N}}$) the mass density of electron-positron pairs will decrease according to $t^\beta e^{-1/t_0}$ for some constants β, t_0 . Note that this is not inconsistent with the fact discussed earlier whereby black holes undergo only a finite number of collisions in infinite time. It is only necessary that an electron-positron pair undergo one collision in its history for annihilation.

At very late times, then, the universe near the $k = 0$ Friedmann will have material consisting of radiation, reactions such as (1) and their reverse, plus the reactions



and the zero-point energy. If we include the zero-point contributions to (1) and (2) in the zero-point energy, we see that reactions (1) and (2) go from left to right—from matter to radiation—because the number of massless particles with energies sufficient to produce an electron-positron pair, say, is proportional to the Boltzmann factor $e^{-1/T}$, and $T \propto t^{-N}$, since the expansion is cooling the ambient radiation. The reaction from left to right, on the other hand, is proportional to the particle number density, which is decreasing according to t^{-N} . Thus at very late time the matter will primarily be radiation and zero-point energy.

The effective stress-energy tensor of the zero-point energy has been recently calculated by Davies *et al.*^{4,5}. For example the vacuum energy density for massless fields in a flat background evolves as $\rho_{\text{vac}} \sim t^{-4}$ and decays as $\rho_{\text{vac}} \sim R^{-8}$ or $\sim R^{-6}$ in radiation and matter dominated universes respectively.

The calculation of a realistic zero-point energy equation of state would involve numerous interacting fields, and would be too difficult to carry out. However, it seems reasonable to assume that the zero-point energy of the interacting fields at very late times in an expanding universe would be that of those particles which would be final state particles in the absence of the zero-point energy. We have argued above that these particles would be massless particles of various types (photons, neutrinos, gravitons), and so the effective vacuum energy would be negligible compared with the radiation background at very late times. Thus a universe neighbouring a flat Friedmann universe will be dominated by radiation at late times.

The dynamical future

We shall summarise some of the assumptions and conclusions of Collins and Hawking and discuss certain other points of the general and technical interest in their calculation before seeing how they are significantly modified in the light of Hawking's subsequent¹⁰ and remarkable discovery of the quantum mechanical evaporation of black holes and accompanying baryon non-conservation¹¹. We have just argued that these processes will ensure that the asymptotic environment of the Universe will be that of a radiation gas in the marginally bound case.

Collins and Hawking were the first to attempt to frame a precise definition of isotropisation for a homogeneous cosmology; a model was said to isotropise if, as $t \rightarrow \infty$, it satisfies the following

- I1: $V \equiv R^3 \rightarrow \infty$, where V is the co-moving volume.
- I2: $T^{00} > 0$ and $T^{0i}/T^{00} \sim v^i \rightarrow 0$, where $T^{\mu\nu}$ is the stress energy tensor; v , velocity field of the matter.
- I3: $\sigma/\theta \rightarrow 0$, where σ is the kinematic shear and $\theta \equiv \dot{V}/V$.
- I4: $\beta \rightarrow \beta_0$, where $\beta \equiv \int_0^t \sigma dt$ and $\beta_0 \equiv 0$.

Condition I1 aims to exclude closed universes (those having compact, spatial three-slices of homogeneity). Another criterion must be used in homogeneous models that recollapse; one proposal³⁹ gauges the isotropisation by measuring the degree of isotropy in the spatial three-curvature at maximum volume. Condition I2 of the Collins-Hawking criteria requires the peculiar velocity of matter motion relative to the surfaces of homogeneity to die away in time, whilst conditions I3 and I4 seek to exclude those models in which the shear distortion decays more

slowly than the universe is expanding since this would build up a large cumulative distortion with increasing time. Collins and Hawking also make use of two general constraints on the matter fields within the cosmologies. The first, the dominant energy condition (DEC)⁴⁰, requires $T^{00} \geq |T^{ab}|$ whilst the second, the positive pressure criterion (PPC)⁴¹ requires $\sum_{k=0}^3 T_{kk} \geq 0$. Although the first of these will be microscopically violated in the quantum processes⁴² described above, it should still be valid 'on the average' or on large spatial scales. Collins and Hawking consider the most general homogeneous perturbations to the flat, ($k=0$), and open ($k=-1$) Friedmann space-times. The most general is of the Bianchi VII_h type requiring one more arbitrary constant to specify the metric than in the type VII₀ case which generalises the $k=0$ isotropic model and is a special case of the VII_h generalisation of an open, isotropic universe. The Collins-Hawking analysis yields:

Theorem 1 (ref. 1): If DEC and PPC are satisfied then the set of homogeneous initial data giving rise to models which approach isotropy is of measure zero in the space of homogeneous data.

That is, as defined by I1-I4 isotropy is in general an unstable property of an expanding universe independent of its equation of state. In proving this theorem they assumed that conditions I1 and I3 held and showed that this implied I3 and I4 could not. This tells us that the inevitable instability revealed by their theorem is associated with a non-decaying shear mode violating I3 and/or I4. In fact, examination of the VII_h model close to isotropy reveals $\sigma/\theta \rightarrow \text{constant}$ as $t \rightarrow +\infty$. Thus, $\beta \sim t$ and an ongoing cumulative distortion arises to first order. There could of course also exist in the VII_h perturbation a vortical instability violating I2 which is explicitly suppressed by the method of proof.

Next, Collins and Hawking fix on a preferred subset of cosmologies—those in the neighbourhood of the zero energy ($k=0$) models and their homogeneous perturbations of type VII₀. In this case the behaviour qualitatively changes since the stability is in fact sensitively dependent upon the particular matter content of the space-time—as might be anticipated since these models, by definition, contain sufficient matter to influence and balance the expansion energy at all times. (In the open VII_h models the asymptotic behaviour is dominated by kinematic and geometrical effects and all memory of realistic matter fields is lost as $t \rightarrow \infty$.) In order to arrive at a strong result regarding zero energy universes CH make the strong assumption that the matter content of the unperturbed isotropic model be just pressureless dust, ($p=0$ state), and that traceless radiation fluids enter only as components of the perturbed matter currents. With this addition they prove:

Theorem 2 (ref. 1): Let S be the subspace of type VII₀ initial data in the space of all homogeneous initial data. If the unperturbed Friedmann model contains dust matter and the perturbed matter content be comprised of dust and zero rest matter with tracefree stress tensor satisfying DEC, then, there exists an open neighbourhood of the $k=0$ Friedmann initial data such that all data in this neighbourhood give rise to models which isotropise.

Thus, if a dust-filled universe lies sufficiently close to a state of zero total energy then it is stable against the development of anisotropy via homogeneous gravitational wave modes concordant with its global topology. (There may, of course, exist additional inhomogeneous gravitational wave perturbations to which it is unstable; see Bonnor^{43,44}, but his models are not general.)

From theorems 1 and 2 Collins and Hawking could draw either of two conclusions. Either: (i) The Universe is young; it is not of zero measure and is presently anisotropising under the action of generic homogeneous perturbations which have had, as yet, insufficient time to grow noticeably out of the linear regime. Or: (ii) the Universe is in the neighbourhood of the zero total energy state, the most general homogeneous perturbations admitted by its geometry are of Bianchi type VII₀ and decay in time. The Universe is thus isotropising but is of zero measure in

the metaspaces of all possible cosmological initial data sets. Collins and Hawking prefer option (ii) and justify its relative unlikelihood by appeal to an anthropic⁶⁻⁸ argument to the effect that galaxies, and hence life supporting environments rich in biologically advantageous heavy elements, would only be expected to arise from such special initial data sets. Conclusion (i) was regarded as unsavoury because it requires our existence as observers to arise in a special interval of cosmic time before the anisotropy has a chance to grow large. Such an objection seems inconsistent since the same 'anthropic' principle used to explain the special binding energy necessary to support (ii) would also defend this no less special assumption required to uphold conclusion (i). Also the arguments invoked to incorporate the Anthropic Principle seem unconvincing given our present knowledge of galaxy formation. For, we might claim, the main problem in explaining the presence of protogalaxies by quasi-statistical fluctuations from homogeneity in the initial state is that these fluctuations do not appear to grow fast enough to give a self-consistent explanation for galaxy formation in any Friedmann universe, whatever its binding energy. To provide understanding of the later nonlinear stages of galaxy formation this difficulty is usually side-stepped and appropriate initial amplitudes simply postulated at some *ad hoc* initial epoch which will guarantee the present structures. Such a procedure really explains nothing of course. *Ad hoc* initial amplitudes will indeed allow flat Friedmann universes to produce bound inhomogeneities by the present epoch as Collins and Hawking wish but although the growth of relative overdensities is exceedingly faster (slower) in closed (open) background universes, the presence of galaxies in these backgrounds can also be explained by initially larger (smaller), but equally *ad hoc*, initial fluctuations.

Before examining the stability of Friedmann universes in more detail, we harmonise two apparently contradictory results, which might otherwise lead to confusion. In an earlier paper Collins and Hawking⁴⁵ also examined the behaviour of homogeneous, gravitational wave perturbations to Friedmann models and deduced the precise constraints placed upon the present shear and vorticity of the Universe by the microwave background isotropy over large angular scales. In performing this analysis they showed there exists a shear distortion mode growing in time ($\sigma/\theta \sim t^{2/3}$) if the Bianchi VII₀ model is linearised about the flat Friedmann universe. This appears to explicitly contradict theorem (2) of Collins and Hawking quoted above in the light of their isotropisation condition 13 ($\sigma/\theta \rightarrow 0$ as $t \rightarrow \infty$). However, a closer examination reveals that neither conclusion need be in error. In this earlier paper Collins and Hawking⁴⁵ discover the following unstable Bianchi VII₀ shear distortion mode to a flat, dust-filled Friedmann model:-

$$\sigma_{32} \sim \int \tau^{-3/2} [DJ_{3/2}(2\tau) + EJ_{-3/2}(2\tau)] d\tau$$

$$\theta \sim t^{-1} \text{ where } \tau \sim t^{1/3} \text{ and } \dot{D} \equiv 0 \equiv \dot{E}; \quad \dot{\cdot} \equiv d/dt \quad (2)$$

To exhibit the short time behaviour of this mode we may use the standard small parameter expansion of the Bessel functions to obtain to first order:-

$$\sigma/\theta \sim At^{2/3} + Bt^{-1}; \quad \dot{A} \equiv 0 \equiv \dot{B}$$

and there indeed appears an anisotropising A-mode of VII₀ type close to the flat isotropic model over short times. However, the presence of the Bessel functions in (3) indicates that the shear mode is oscillatory over large time intervals. Using the boundedness of the oscillatory component we do indeed find the VII₀ model ($p=0$), to tend asymptotically to the flat Friedmann model over large times and

$$\sigma/\theta \sim \alpha t^{-2/3} + \beta t^{-1}; \quad \dot{\alpha} \equiv 0 \equiv \dot{\beta} \quad \text{for } p=0.$$

The short time expansion used in ref. 45 thus traces the initial growing phase of an oscillatory but asymptotically decaying function.

We now turn to consider the new consequences for the Collins-Hawking analyses from our arguments above regarding

the inevitable asymptotic radiation domination of a marginally bound universe in the far future because of baryon non-conservation by the gravitational interaction. This significantly alters the conclusions drawn from theorem (2) of Collins and Hawking regarding the asymptotic dynamical state of universes close to zero total energy undergoing generic homogeneous perturbations. These universes will no longer isotropise, but in general will become vortically unstable at late times. To understand intuitively why this is so consider the newtonian dynamics of a ball of expanding fluid with radius R subject to a small rotational perturbation of angular velocity ω . The angular momentum of this expanding ball is given by $J \equiv I\omega \sim MR^2\omega \sim (\rho R^3)R^2\omega$. Now for a perfect fluid with equation of state $p = (\gamma - 1)\rho$, conservation of mass requires $\rho \sim R^{-3\gamma}$ and thus $\dot{J} = 0$ requires $\omega \sim R^{3\gamma-5}$ with the associated linear velocity evolving as the ball expands as $v \sim R\omega \sim R^{3\gamma-4}$. Thus for newtonian ever-expanding universes filled with radiation ($\gamma = 4/3$) the velocity, v , approaches a constant value, violating isotropisation condition 12 of Collins and Hawking. However, this is in fact, physically, a growing instability. Inspection of the Raychaudhuri equation^{46,47} governing the general kinematics of the fluid ball is (in the relativistic or newtonian case):

$$3\frac{\ddot{R}}{R} = 2(\omega^2 - \sigma^2) + \nabla_a \cdot A_a - \frac{1}{2}(\rho + 3p)$$

(where A_a is the fluid acceleration), showing that the dynamical influence of the vorticity is gauged by the contrast parameter $\omega^2\rho^{-1}$ which expresses the ratio of the centrifugal energy driving distortions to the isotropy-inducing matter stresses. We have, for small anisotropies, $\omega^2\rho^{-1} \sim R^{9\gamma-10}$. So, for a radiation fluid, (just $p \geq \rho/9$ on average suffices), a growing vortical instability develops as the fluid expands which will create a pancake-like or cigar-like configuration and rapidly increasing deviation from sphericity when $\omega R/\dot{R} \sim 1$. A newtonian universe with near radiation matter content would become increasingly dominated by the energy of random and rotational motions rather than the inertia of its rest-mass content. This is in complete contrast to the fate of a dust, ($\gamma = 1$), ball where the vortical effects die away as $\omega^2\rho^{-1} \sim R^{-1}$ with the expansion. We shall confirm this behaviour with a fully relativistic derivation below, in which case the ball of fluid will be infinite. This simple argument also exhibits certain weaknesses of the CH isotropisation conditions. For instance, condition 12 allows a model to be classed as isotropising if $v \rightarrow 0$ as $t \rightarrow \infty$ even though $\int v dt \rightarrow \infty$ and thus the cumulative distortion from the velocity or vorticity perturbations leads to an aspherical configuration. We really require a stronger criterion: that the cumulative effect of the vorticity is non-increasing. By analogy with I4, a possible criterion would be

$$\Omega \equiv \int_0^t \omega dt \rightarrow \lambda \text{ as } t \rightarrow \infty; \dot{\lambda} \equiv 0.$$

For example, in a universe close to the flat, Friedmann model $R \sim t^{2/3\gamma}$, and conservation of angular momentum indicates that $\Omega \sim t^{(9\gamma-10)/3\gamma}$. Vortical effects will not lead to cumulative distortions if $\gamma < 10/9$ in agreement with the other criterion used above to discuss vortical instability, namely that $\omega^2/\rho \sim (\omega/\theta)^2$ decay as $t \rightarrow \infty$ for isotropisation, (this condition is analogous to condition I3 on the shear). These results indicate that there exist homogeneously expanding newtonian universes which may become increasingly far from isotropy as $t \rightarrow \infty$ due to their vortical instability and yet be classified as 'isotropising' by the Collins-Hawking criteria. Furthermore, flat newtonian universes may exhibit vortical instability whenever their fluid content satisfies $p > \rho/9$ and in particular radiation dominated universes are unstable.

It can be demonstrated that this vortical instability arises also in relativistic cosmology, as one might anticipate, since it is a consequence only of a fundamental invariance principle—the conservation of angular momentum. For concreteness we consider the homogeneous Bianchi VII₀ model (which general-

ises the flat Friedmann model) filled with radiation to demonstrate the instability; but the instability exists for all $p \geq \rho/9$ fluids. By vorticity we shall mean the rotation of matter about an observer moving with the matter, relative to an inertial frame defined by gyroscopes. The peculiar velocity will represent the velocity of the matter relative to the surfaces of constant homogeneity. We use the notation of Collins and Hawking¹ with our $R \equiv e^a$ and $\rho \equiv \mu$ as scale factor and density.

A homogeneous cosmological model is defined as one which admits a three-parameter group of isometries, simply transitive on a family of space-like hypersurfaces⁴⁸. Collins and Hawking¹ label these surfaces by t chosen so that $g^{\mu\nu}t_{;\mu}t_{;\nu} = -1$ and the metric has the form $g_{\mu\nu} = -n_\mu n_\nu + g_{AB}E_\mu^A E_\nu^B$ where $g_{AB} \equiv R^2(e^{2\beta})_{AB}$ with β trace-free and $n_\nu = -t_{;\nu}$ is normal to the surfaces of homogeneity (Greek indices run 0–3 and Latin 1–3). Thus, spatial homogeneity implies $\rho_{;\nu} = -\rho n_\nu$ for a scalar, ρ . Consider a radiation fluid with

$$T^{\mu\nu} = \frac{4}{3}\rho U^\mu U^\nu + \frac{1}{3}\rho g^{\mu\nu}$$

The conservation equation $T^{\mu\nu}_{;\nu} = 0$ yields

$$[\rho(U^0)^{4/3}R^4]' = -\frac{4}{3}\rho(U^0)^{1/3}C_{AB}^A U_C R^2(e^{-2\beta})_{BC} \quad (4)$$

where C_{AB}^A are the structure constants of the homogeneous, space-group symmetries. The acceleration by the fluid pressure is given by another conservation equation as

$$\frac{4}{3}\rho U^\mu_{;\nu} U^\nu = -\frac{1}{3}\rho_{;\nu} h^{\mu\nu} \quad \text{where } h^{\mu\nu} = g^{\mu\nu} + U^\mu U^\nu$$

so:

$$[\rho^{1/4}U_A]' = \rho^{1/4}(U^0)^{-1}C_{CA}^B U_B U_D R^{-2}(e^{-2\beta})_{CD} \quad (5)$$

The components of the vorticity vector are defined as $\omega^\mu \equiv \eta^{\mu\nu\lambda\rho} U_\nu U_{\lambda;\rho}$ where $\eta^{\mu\nu\lambda\rho}$ is the alternating tensor. Thus,

$$\omega^A = R^{-3}\epsilon_{ABC}[\frac{1}{2}C_{BC}^D U_D U^0 + U_B \dot{U}_C] \quad (6)$$

and

$$\omega^0 = \frac{1}{2}R^{-3}\epsilon_{ABC}[C_{BC}^D U_A U_D]. \quad (7)$$

The Bianchi VII₀ model generalising the flat Friedmann universe, is of Behr-Ellis-MacCallum^{49,50} class A having $C_{AC}^A = 0$. Thus, from (5) for small β , close to isotropy we have:-

$$(\rho^{1/4}U_A)' \sim \rho^{1/4}C_{CA}^B U_B U_C R^{-2}$$

(4) implies $\rho \sim R^{-4}$ thus,

$$(R^{-1}U_A)' \sim R^{-3}C_{CA}^B U_B U_C \sim R^{-3}[1 + O(v^2)]$$

Thus to first order, perturbations in velocity and vorticity of this type evolve from the Friedmann background as $U_A \sim R$ giving the three-velocity squared as $v^2 \sim U_A U^A \sim \text{constant}$ and to first order for the vorticity vector, from (6) and (7) we have,

$$\omega^A \sim R^{-3}[\text{const. } R + RR] \sim R^{-2}$$

$$\omega^0 \sim R^{-3}[\text{const. } R^2] \sim R^{-1}$$

Thus, the vorticity scalar evolves $\omega^2 \sim R^{-2}$ in agreement with the simple newtonian arguments given above. Modifications due to nonlinear coupling with the shear are to be expected when the vorticity becomes large. Therefore, the flat, radiation-filled Friedmann universe will be unstable to the development of vorticity; this behaviour is not an artefact of the homogeneity of the perturbations and may be recovered from studies of inhomogeneous vector perturbations to isotropy⁵¹. Intuitively, we may see this from Raychaudhuri's equation which shows that when baryons and leptons have been eliminated by the mechanisms described in the last section the terms driving the expansion are $\rho_\gamma \sim R^{-4}$, the shear $\sigma^2 \sim R^{-6}$ and the rotational energy $\omega^2 \sim R^{-2}$ in the linear regime. For large R the rotational energy clearly dominates. In models with open spatial sections ($k = -1$), (type VII_k being the most general homogeneous example containing the Friedmann, open model), such an effect also competes with the spatial curvature terms $\sim R^{-2}$ and the relative strengths of the curvature and rotation terms will determine the asymptotic behaviour. If the curvature effects

dominate as $R \rightarrow \infty$ they will suppress the vortical instability and allow the shear instability $\sigma/\theta \rightarrow \text{constant}$ found by Collins and Hawking to control the future evolution. However, it is still possible for the spin to significantly determine the final state via its non-linear interaction, see for example refs 52, 53. This completes the argument that the 'heat death of the Universe' is sufficient to ensure all isotropic universes are unstable irrespective of their binding energy. The fate of perturbations to closed Friedmann universes was not considered by Collins and Hawking in ref. 1 but other studies indicate that the general homogeneous closed universe of Bianchi type IX does not 'isotropise' in any meaningful sense³⁹ as it approaches maximum volume. For instance, the spatial curvature does not tend to directionally equalise and will produce anisotropy in the Hubble rates.

Another conceivable cosmological artefact of eschatological significance is the possibility of a non-zero cosmological constant Λ whose presence introduces terms of the order of ΛR^{-2} in the energy evolution equations. This is of the same order as the vortical energy term ω^2 asymptotically in the radiation-dominated universe. We might thus suspect that in the $\Lambda > 0$ case, allowing ever-expanding universes, the isotropic stress induced by the presence of this hypothetical cosmic force field might be of sufficient magnitude to dominate the vortical energy. A negative Λ would essentially reproduce the closed universe evolution since it has been shown⁵⁴ that a space-time with $\Lambda < 0$ cannot expand forever: all timelike curves have a finite length $< 2\pi(3/|\Lambda|)^{1/2}$.

It is possible that the collective gravitational effects of density perturbations could be the predominant agent in driving the cosmological flow from homogeneity and isotropy in the large at late times. For example, an irregularity spectrum $\delta\rho/\rho \sim M^{-\alpha}$ contributes to the metric distortions as $\delta g/g \sim M^{(2/3-\alpha)}$. The preferred $\alpha = 2/3$ Zeldovich spectrum^{32,55} is envisaged with metric perturbations constant on all scales as they enter the horizon. If a 'white noise', $\alpha = 1/2$, spectrum prevails then they would eventually dominate the dynamics as $M \rightarrow \infty$ and couple with the vortical motions to determine the asymptotic behaviour. It is not known, of course, if such spectra really do extrapolate to indefinitely large mass scales.

We have in this section completed a proof that physically realistic ever-expanding universes asymptotically deviate from isotropy—eternity is unstable.

Asymptotia

The future state of non-flat, ever-expanding universes is characterised¹ by a cumulative shear distortion in the expansion flow. That of the ever-expanding flat models is analogous to a relativistic vortex, for the dominant instability is due to the vorticity of the matter flow lines. In both cases the final state is characterised by increasing irregularity. However, these irregularities will not in general result in a breakdown of the global causal structure in the large; a generic homogeneous space-time is globally hyperbolic, which means that the future is determined by the initial data on some space-like hypersurface. Siklos has shown⁵⁶ that homogeneous space-times which violate global hyperbolicity have two fewer free parameters than the most general spatially homogeneous space-times. He also showed that type VII₀ space-times which satisfy $R_{ab}K^aK^b > 0$ for all null vectors K^a cannot violate global hyperbolicity, and this inequality would hold in a VII₀ space-time which is dominated by randomly orientated radiation.

Although global hyperbolicity will hold on a large scale, it will be violated in the small in an ever-expanding universe. The complete evaporation of a black hole will generate a naked singularity⁵⁷ and this necessarily means a violation of global hyperbolicity. Thus, if one considers global hyperbolicity—the ability to predict the future—to be a property which must hold, even in the small, in any physically realistic space-time, then one is forced to conclude that the actual Universe is closed. Only in this case can an all-encompassing, final singularity intervene to prevent the inevitable formation of naked singularities from black hole evaporation.

The matter vorticity couples to the Weyl tensor in the second order⁵⁰, so the instabilities we have discovered and those of Collins and Hawking¹ imply that the Weyl tensor grows with time. Now Penrose has suggested^{58,59} that the entropy of the gravitational field is proportional to "some suitable integrated measure of the size of the Weyl curvature". If this connection between the gravitational entropy and the Weyl tensor holds, then our results can be interpreted as saying that the entropy of the gravitational field increases with time. This is just what we would expect; the entropy of all fields should increase with time, unless the decrease of the entropy of one field is more than counter-balanced by the increase in the entropy of another. An asymptotic approach to a Friedmann space-time, which is the future behaviour of the universe envisioned by many cosmologists—in particular by Collins and Hawking¹—would constitute a decrease in the gravitational entropy without a corresponding increase in the entropy of the other fields. The second law of thermodynamics tells us that this cannot happen (statistically speaking, it must be very improbable) and this is what we have found.

We have arrived at a final state for an ever-expanding universe which is somewhat different from the "state of eternal rest" pictured by the nineteenth century physicists. In our view the space-time geometry is becoming more and more irregular at very late times. Both pictures envision an asymptotic approach to a state of maximum entropy; our version of the heat death is different because we have included the gravitational entropy.

Time is a measure of change, and both versions of the heat death have fewer and fewer changes as asymptotia is approached. Thus, one might expect that physical time could slow down relative to proper time in the far future. Although the asymptotic state is reached only after infinite proper time in an ever-expanding universe, it is possible that it would be reached in *finite* physical time. Misner has argued⁶⁰ that proper time is an inappropriate measure of physical time near a singularity. Because changes occur with great rapidity (as measured in proper time), Misner contends that the singularity should have occurred in the infinite past as measured in physical time, though only about twenty thousand million years ago in proper time. A mathematically appealing timescale which has this 'Misner time property' of placing singularities at temporal infinity is the so-called York extrinsic time^{24,61} York⁶¹ has proposed foliating a space-time with spacelike hypersurfaces of constant mean extrinsic curvature, and defining time to be given by $T = -\frac{4}{3}K^a_{ab}$, where K^a_{ab} is the extrinsic curvature of the hypersurface. ($K^a_a = K^1_1 + K^2_2 + K^3_3$ is the mean curvature: the sign is chosen as to make T increase to the future, and the factor $4/3$ simplifies the expression of the dynamical Einstein equations). Such a foliation can be shown to exist in a fairly general class of closed universes⁶²; and in closed universes, the foliation will also be unique^{62,63}. If this foliation by constant mean curvature hypersurfaces exists and is unique in open universes also, then it would define an absolute time for the Universe. In ever-expanding universes with an initial singularity the York time varies between $-\infty$ at the singularity and zero at asymptotia. Hence if York time measures physical time in the far future, then an ever-expanding universe, which exists forever in future proper time, will continue to exist for only a finite physical time. Vaguely speaking, only a finite number of changes will occur in the future; the heat death puts an end to change.

Note that in closed universes, which have both initial and final singularities, York time varies⁶² between $-\infty$ and $+\infty$. Closed universes thus have a finite future in proper time, but an infinite future in York time. If York time is indeed the appropriate measure of physical time near the final singularity, then the infinite future duration of a closed universe can be interpreted as saying that the gravitational field provides an unlimited source of free energy near the final singularity, and this permits an infinite number of changes—physical time intervals—to occur.

We close with a final speculation. One of the fundamental problems of statistical mechanics is accounting for the global

increase of entropy without introducing a very strong stochastic assumption, such as the postulate of random phases or the assumption of coarse-graining, which is difficult to justify. As discussed in detail by Brush¹⁹, the justification of this type of stochastic assumption has a subjective flavour about it. Coarse-graining, for example, generally requires an appeal to human inability to measure a difference between certain classes of states⁶⁴. What is required is a proof of entropy increase which does not require such a stochastic assumption but uses only the equations of motion of the existing fields and particles (all time-reversible except for the super-weak *T*-violating interaction) and the universal initial conditions. Penrose's suggested connection between gravitational entropy and the Weyl tensor

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may provide a mechanism for such a proof. The present article and the work of Collins and Hawking indicate (but do not prove) that in homogeneous ever-expanding universes, the Weyl tensor will increase as one moves far away from a generic, conformally flat singularity. Thus, the increase of Penrose gravitational entropy follows from assumptions about the Initial Conditions: the initial singularity is both generic and conformally flat. Is the Penrose entropy the ultimate source of all entropy increase?

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Punctuated evolution of tectonic style

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A continuous secular evolution in the vigour of mantle convection may have translated into a series of distinct stages in crustal tectonic style.

THERE is much debate about the nature and relative magnitude of the forces responsible for the motion of lithospheric plates¹. The possibilities considered include the viscous drag of convecting asthenosphere and the gravitational push away from oceanic ridges, but it is the gravitational pull, or negative buoyancy, of the cold, subducting lithospheric slab which emerges as a major^{2,3}, if not the predominant⁴⁻⁶, force. The age of crust being subducted today ranges from 5 to 175 Myr (ref. 6) and Atlantic oceanic crust ~200 Myr old has not even started, so clearly achieving a critical value of negative buoyancy is not the only cause of subduction. Some combination of these forces, and perhaps circumstances such as convergence and over-riding of ridge by continent, must be enough to trigger subduction, and cause the spectrum of subducting-crust ages. Nevertheless, if it is accepted that on a world-wide basis, negative buoyancy is the major force in this process, then this view has important ramifications with respect to Precambrian tectonic style.

Buoyancy

From a plate-tectonic point of view, buoyancy is measured relative to the average density of the asthenosphere. Crust, whether it is basaltic or granitic, is positively buoyant, whereas the cold peridotitic part of the lithosphere is denser than the hot asthenosphere. Overall negative buoyancy of lithosphere results from the cooling of oceanic plates as they recede from ridges, and the denser, mantle part of the lithosphere thickens relative to the thin positively buoyant crust.

If higher average heat flow implies a thinner lithosphere, then negative buoyancy must have been less important in the past. Consideration of the possible previous magnitude of the terrestrial heat flux⁷, forces associated with earlier, more vigorous convections⁸, and their gradual temporal evolution, forms the basis for the speculation developed below, that three critical stages in the evolution of tectonic style on Earth may have occurred: (1) initially any thin crust formed was completely recycled by viscous drag forces associated with rapid convective circulation. (2) As these forces diminished, crust accumulated like scum, possibly to form a globe encircling layer. As such crust thickened by continuous outpouring of lava and tectonic

compression, it would have been recycled by remelting at the base. Archean terrains have features compatible with this hypothesis. (3) Further decrease in heat flux and increase in negative buoyancy culminated in subduction of relatively thinner segments of this crustal scum, initiating plate-tectonics, and development of the present continent-ocean dichotomy. The essential details of this argument are as follows.

Continent-ocean dichotomy

Dissipation of up to 45% of the contemporary heat flux is attributed to the creation and cooling of new oceanic lithosphere⁹. The balance, produced by some combination of radioactive decay within the lithosphere (the upper continental crust in particular¹¹) and a sublithosphere flux is conducted to the surface. (Although thought necessary to explain the observed heat flow values, the source of the flux is not clear. It could

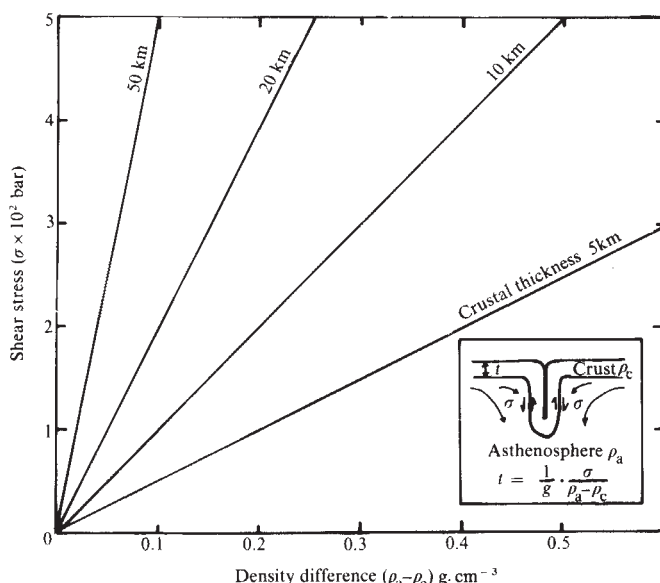


Fig. 1 Thickness of positively buoyant crust which would be in equilibrium with an asthenosphere-imposed downward-dragging shear stress (see inset). Curves for various crustal (or lithospheric) thicknesses are plotted as a function of shear stress and positive density difference ($\rho_{\text{crust}} > \rho_{\text{asthenosphere}}$). $t = (1/g)(\sigma/(\rho_a - \rho_c))$.

be associated with the small-scale convection of McKenzie and Weiss⁸, or Richter and Parsons¹², or be generated by viscous dissipation associated with convective flow, as suggested by Schubert *et al.*¹³.) The manner of dissipation of the significantly higher Precambrian heat flux^{7,8} is becoming a focal point of the debate concerning Precambrian tectonic style¹⁴⁻¹⁶ and whether or not plate tectonics were operating¹⁷. The more conventional uniformitarian view assumes that the mechanism involving the creation, cooling and subduction of new lithosphere, which accounts for 45% of today's flux, must have been at least proportionately more vigorous in the past¹⁸. In this view a greater length of spreading ridge, or faster spreading, is required to accommodate the higher flux. But this cannot be the case if negative buoyancy is the critical or dominant component of the plate driving force. Faster spreading would imply less time between ridge and trench, and greater ridge length would imply less distance between ridge and trench; in either case less cooling and hence less negative buoyancy could be achieved.

With respect to contemporary plate tectonics, buoyancy-powered subduction can occur as soon as the average density ($\bar{\rho}$) of the lithosphere is greater than the underlying asthenosphere. Basalt ($\rho = 3.0$)¹⁹, by itself, is positively buoyant with respect to the asthenosphere ($\bar{\rho} \sim 3.22$); it is the growth of cold mantle lithosphere ($\bar{\rho} \sim 3.30$) which increases the average density and eventually renders the lithosphere negatively buoyant. If, as has been claimed²⁰, the residual periodotite from which the basalt

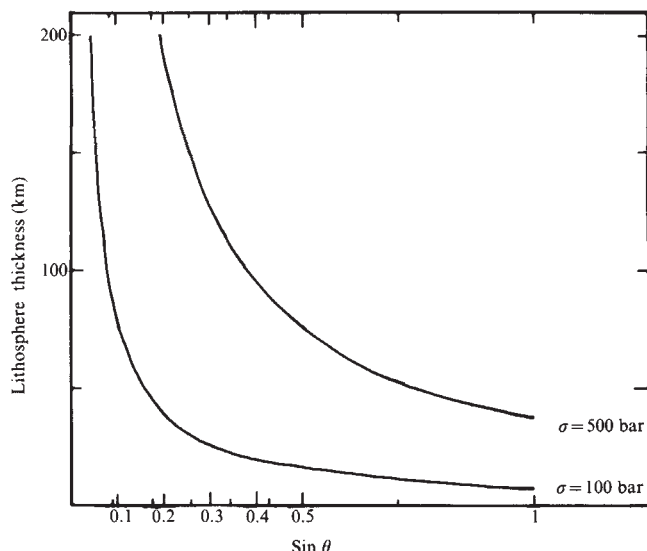


Fig. 2 Differential shear stress (σ) which would be generated along the Mohorovic discontinuity as a function of inclination or tilt (θ) of this boundary and total lithosphere thickness. Differential stress arises from positive buoyancy of crust relative to negative buoyancy of cold mantle part of the lithosphere. Model assumes lithosphere, isostatic with asthenosphere ($\rho_a = 3.22$) and consisting of 17.7% crust ($\rho_c = 2.85$) above mantle ($\rho_m = 3.30$). $t_{\text{lithos}} = 7.6\sigma/g \sin \theta$.

was derived is also positively buoyant, then even more cooling and growth of lithosphere is required to achieve negative buoyancy.

The average magnitude of the negative buoyancy required to trigger buoyancy-powered subduction may be approximated in terms of the average lithosphere subducting today which is about 80 Myr old, 75 km thick, and has a heat flow of $\sim 60 \text{ mW m}^{-2}$ (1.4 HFU)²¹. If twice this heat flow (120 mW m^{-2} , $\approx$ lithosphere 20 Myr old, ~ 40 km thick) is taken as the maximum commensurate with buoyancy-powered subduction, then there was a time earlier in Earth history before which it was unlikely to occur. Even using the cooler (relative to a chondritic Earth) extrapolated heat flow curve of Wasserburg *et al.*⁷, the average surface heat flow from the Earth was in excess of (120 mW m^{-2}) until $\sim 1,500$ Myr after the Earth formed⁶. On this basis subduction, powered primarily by negative buoyancy forces, could not have started until relatively late in Earth history.

Viscous drag

Although the lithosphere was likely to have been thinner when heat flow was higher, the viscous drag of more rapidly circulating asthenosphere would certainly have been stronger. Assuming that the Earth was hot enough for mantle-wide convection soon after its formation, McKenzie and Weiss²² conjectured that originally this viscous drag force may have been as much as

Table 1 Correlation between tectonic sequence and Earth history

Tectonic sequence	Date (Myr BP)	Stages in Earth history
Viscous drag subduction	→ ~ 3.6 by	Complete cycling of crust Archaean granite— greenstone terrains are remnants
Scum—crust	3.6 → ~ 2.6 by	
Buoyancy powered subduction		
(1) Decoupling of mantle lithosphere from crust	2.6 → ~ 0.6 by	Cause of Proterozoic intracratonic mobile belts
(2) Complete lithosphere subduction	2.6 → 0	Growth and maintenance of ocean: continent crustal dichotomy

$5 \times 10^7 \text{ Nm}^{-2}$ (500 bar). This, they pointed out, would have been more than sufficient to fracture the lithosphere or crust. At this time, however, the lithosphere may well have been so thin as to consist of little more than positively buoyant basaltic crust floating on asthenosphere. Being gravitationally stable, such a lithosphere would resist subduction, and it would thicken by overthrusting above the downwelling convective zone. It is possible, however, that the viscous pull of downwelling asthenosphere could effectively drag even positively buoyant thin crust (or depleted mantle lithosphere fragments) back into the mantle. The relationships among viscous drag force, density contrast and lithosphere thickness necessary for this to occur are shown in Fig. 1. Even though the model used is oversimplified, it shows, for example, that lithosphere slabs 10 km thick with an average density contrast of $+0.25 \text{ g cm}^{-3}$ could be dragged down by a shear stress of ~ 250 bar. On these terms, it can be claimed that the early Earth may have consisted of a mosaic of small, thin plates, circulating with the vigorously convecting asthenosphere beneath, a model similar to that proposed by McKenzie and Weiss⁶.

As heat flow declined, the velocity of the circulating asthenosphere and the associated viscous drag force would presumably decrease. With slower spreading, however, the average age of the plates would increase, the mantle component of the lithosphere would become thicker and the average positive $\Delta\rho$ would decrease, eventually becoming negative. All the latter changes would make the lithosphere more subductible. Thus subduction driven by viscous drag forces may well have evolved continuously into buoyancy-powered subduction such as we have today.

Discussion

Two aspects of this simple evolution, however, merit further consideration. First, before the onset of buoyancy-powered subduction, a stage may have intervened when the viscous drag was insufficient to pull down positively buoyant lithosphere. As a result, crust may have accumulated like scum until, conceivably, the whole Earth was covered. Recycling of this crust would have occurred because thickening by tectonic compression and continued outpouring of lava on the surface would inevitably have led to remelting at the base. Eventually, however, the right combination of thin crust, relative to thick mantle lithosphere, necessary for critical negative buoyancy, would have been achieved somewhere on the globe, and buoyancy-powered subduction would have commenced. Complementary spreading would form protooceanic crust, and the growth of the ocean-continent crustal dichotomy could have begun. Second, cooling and thickening of mantle lithosphere beneath thicker crust could lead to their decoupling and independent 'subduction' of the former. Decoupling of lithosphere from crust would require that the shear stress on the Mohorovicic discontinuity exceed some critical value.

Such a differential shear stress could arise from the intrinsic positive buoyancy of crust relative to the negative buoyancy of

the cold mantle lithosphere below. As long as the density gradient was perpendicular to the gravitational force (that is, the Moho was horizontal), however, there would be no differential shear stress. But if the discontinuity became tilted, a shear stress would be developed which might suffice for decoupling. Assuming that the average lithosphere was isostatic with respect to asthenosphere (that is, $\bar{\rho}_l = \rho_a$), then the relationship between lithosphere thickness and tilt of the Moho needed for decoupling, for two critical shear stresses, is shown in Fig. 2. With a 25-km thick crust, a tilt of 6° would cause a Moho shear stress of 100 bar; such a tilt would be associated with a warp of half amplitude 15 km and a half-wave length approaching 300 km. When heat flow was higher, the temperature at the Moho was higher, and the critical shear stress for decoupling was less. Decoupling by this means, therefore, seems feasible. Stresses associated with such independent subduction of mantle lithosphere slabs, decoupled from crust above, may have been responsible for the deformation in intracratonic mobile belts common in Proterozoic terrains²³. Today, perhaps, with subcratonic Moho temperatures averaging 500°C , and thicker lithosphere, the tilts and shear stresses necessary for decoupling cannot be achieved.

The degree of validity of the simple relationships proposed here is not known. Even if they are pertinent, the temporal evolution of the magnitude of the parameters involved in the relationships is also unknown, but the trend with the inescapable decline in heat flux must be correct. As Table 1 shows, the appeal of the tectonic sequence postulated lies in the possible correlation with the observed major stages in Earth history.

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Injection events in ocean history

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The reconnection, with the world ocean, of temporarily isolated ocean basins results in injection either of hypersaline waters favouring abyssal stratification and stagnation, or of brackish waters from high latitudes leading to a low salinity surface layer. Mass extinctions and sudden climatic change are closely associated with Arctic injection.

SEVERAL major palaeoceanographic events have become well defined recently through the results of deep-sea drilling^{1,2}. We propose here that several of these events are manifestations of the same type of occurrence, that is, the injection into the world ocean of saline or of fresh water from a temporarily isolated large basin. We first enumerate the events, and then consider the evidence for isolated basins and the timing of opening, the

physical processes governing the injection, and the compatibility of our model with published data on isotopes and carbonate preservation. We also present the first detailed stable isotope stratigraphy across the Cretaceous–Tertiary boundary and interpret it in terms of the injection model.

Major events of the past 100 Myr

The phenomena to be explained are: (1) the mid-Cretaceous event or series of events (Aptian–Turonian) which produced worldwide changes in carbonate deposition² and which brought notable changes in marine biogeography, especially that of reefs, bivalves and nannofossils^{3–6}; (2) the Cretaceous–Tertiary boundary event which brought about great changes in plankton assemblages^{7–14}; (3) the Eocene–Oligocene event, which is marked by a sharp decline in diversity of plankton^{15–17}, a change of benthos¹⁸, a pronounced lowering of the CCD^{19,20} and by a sudden drop in the temperature of deep waters^{21–23}; (4) the Miocene–Pliocene boundary which is marked by diversity decline of planktonic foraminifera^{15,16} and by cooling²⁴ and Antarctic ice expansion²⁵, and which heralds the onset of northern glaciations^{21,25,26}.

Many explanations have been proposed for these events, especially for the Cretaceous–Tertiary boundary, which produced the most profound changes in evolutionary history during the past 200 Myr; these hypotheses are reviewed elsewhere^{27,28}. Here we ask whether these events might be linked by a common oceanographic phenomenon: exchange with a previously isolated basin. Such an exchange was recently proposed for the Cretaceous–Tertiary boundary²⁹.

Recent palaeogeographic maps³⁰ show that preceding each major event a large isolated basin existed whose exchange with the world ocean was restricted to a more or less severe degree. In the Aptian, salt was deposited in a restricted proto-South Atlantic³¹. The same basin received highly carbonaceous sediments in late Aptian to Albian time^{2,32}, showing its restricted status. In the Maastrichtian, the Arctic Ocean was essentially isolated with both the Labrador Passage and the Greenland Passage closed^{30,33}. In the Eocene, a similar situation prevailed, with the Labrador Passage and the Norwegian–Greenland seas closed³⁰. In the early Miocene the western Tethys became isolated by closure of Africa and Eurasia²⁶ and in the late Miocene it went dry intermittently and accumulated large salt deposits^{34–37}.

In all four cases, the restricted basins seem to have opened for full exchange with the world ocean at about the time of crisis, and perhaps exactly coincident with it. Assuming that this is more than a coincidence, are there physical reasons that such an event should lead to worldwide biological and geochemical changes?

We think there are: the global heat budget requires polewards transport of latent heat, especially with warm climate in high latitudes³⁸. Thus, each of the restricted basins is likely to be either more saline (proto-South Atlantic, Mediterranean) or fresher (Arctic) than the world ocean. A sudden onset of exchange, therefore, would upset the existing global density stratification, with profound effects on ecological stability and fertility of the ocean.

In principle, a strong influx of heavy (very saline and/or very cold) water will isolate abyssal waters from overlying intermediate and shallow waters, by increased density stratification. Likewise, a strong influx of fresh water or very warm water, will isolate the upper ocean layer from the waters at depth. However, the exact effect of a catastrophic influx depends on the existing stratification. If, for example, a widespread oxygen minimum exists, influx of heavy water can instantaneously lift the minimum towards shallower depths, provoking a global upwelling phenomenon (the surface water withdraws into the newly opened basin). Other sequences can be readily envisaged; for example, with intruding layers becoming sandwiched between existing ones and thus decreasing stability rather than increasing it. We are concerned here, however, with injection of

waters of extreme density difference, which sink to the bottom or float on top of the ocean.

Cretaceous anoxic events

In the case of the mid-Cretaceous period of anoxic events^{31,39,40} we postulate that the stability of the water column was greatly increased, first in the North Atlantic and later—and sporadically—in the entire Pacific by saline outflow to the north from the early South Atlantic starting in the Aptian. These brine injections reinforced haline stratification in an already salinity-layered world ocean with oxygen-poor and carbon dioxide-rich deep waters^{2,41}. Deep water exchange between North and South Atlantic may have begun sometime in the Cenomanian, shallow-water exchange of normal salinity having been demonstrated for at least as early as the late Albian⁴².

We envisage the mid-Cretaceous South Atlantic as being filled with heavy saline waters, to a level commensurate with the Walvis–Rio Grande sill. These saline waters were produced on surrounding partially flooded shelves and possibly also from the dissolution of pre-existing exposed Aptian salt deposits. The South Atlantic would have acted as a source of heavy bottom water for some considerable time, with pulsing supply, in response to climatic variation on the shelves. This pulsing would have translated into intermittent anaerobism, as reported by Lancelot, Seibold *et al.*⁴³. Supply of nutrients and preservation of organic matter in the Atlantic were provided by estuarine circulation^{44,45}. The South Atlantic surface and intermediate waters were estuarine with respect to the southern ocean, with intermediate cold waters moving north on top of the saline bottom layer and upwelling in the basin. This intermediate water brought the austral faunas described by Premoli Silva and Boersma⁴⁶. The North Atlantic was estuarine with respect to the Pacific with intermediate cold waters moving east on top of the saline deep layer, and subsequently upwelling especially in the eastern Atlantic basin. Both a broad oxygen minimum and bottom-near oxygen deficit resulted, which lasted through Turonian time^{2,47}. The oxygen minimum also reached into adjacent shelf sea areas, by estuarine shelf circulation, generating “tremendous areas of well laminated, dark organic rich shale”⁴⁸ deposited in conditions unfavourable for benthic foraminifera⁴⁹. At the foot of the eastern continental slope, the abyssal saline layer favoured the precipitation of dolomite, and, through the accumulation of CO₂ of ankerite and siderite, as described from Site 138⁵⁰. The mid-Cretaceous anoxic period ceased (for the deep sea) when the South Atlantic ceased to be restricted but became a throughway for austral cool deep waters. These waters were able to mix with the tropical saline inflow because of their similar densities.

Cretaceous termination

In the case of the Cretaceous termination^{11,28}, exchange with a previously isolated Arctic Ocean filled with fresh or very low salinity water has been postulated²⁹. The isolation occurred during the latest Cretaceous regression. On tectonic opening of the Labrador Passage, the light water of the Arctic would be readily displaced by the surficial salt water of the North Atlantic. Assuming from palaeogeographic maps³⁰ and from present conditions that the volume of the Arctic is about 1.3% of that of the world ocean, and assuming complete isolation, we obtain a layer of fresh water some 53 m thick, as a maximum estimate. Mixing this layer to one-half the normal salinity makes a layer over 100 m thick. Hence it includes the entire photic zone. The salinity would be such that open ocean plankton (plus its trophic dependents and stenohaline shallow water benthos) could not survive, as observed^{7,9,10,14,27,51}. Coastal plankton, being less sensitive to lowered salinity⁵² and perhaps certain abyssal plankton, such as deep-living radiolarians, would be much less affected. Likewise, deep-living benthics would not be affected. Indeed, deep-living benthic foraminifera do not show unusual changes at the boundary (refs 53, 54 and L. Tjalsma, personal

communication.). The ensuing short-term climatic changes would affect land animals and to a lesser degree land plants⁵⁵.

Once the unfit planktonic species had been eliminated, successive exchange events (from slight changes in sea level or in sill tectonics) produced intermittent Arctic injections and blooms of opportunistic survivors. Such blooms are well known from the earliest Tertiary, involving both *thoracosphaeres* and *braarudosphaeres*^{13,14,56-60}. When the Labrador Passage had widened sufficiently⁶¹, isolation of the Arctic ceased and so did injection of fresh water. Radiation of plankton species followed in the late Palaeocene¹⁵⁻¹⁷.

Eocene termination

In the case of the Eocene–Oligocene boundary we again postulate late exchange with a previously isolated Arctic, the isolation being produced by regression and by closing the Labrador Passage sometime during the Eocene³⁰. This time the opening is through the Greenland Sea Passage⁶². Again, we assume the Arctic was fresh or had very low salinity, on the basis of the requirement that latent heat must be brought into this polar area in sufficient amounts to keep it ice free. The Eocene termination did not compare with the Cretaceous one in severity; we ascribe this to a more gradual water exchange, to the lack of a maximum exchange event, to a moderate brackishness of the Arctic at the end of the Eocene, or a combination of these. In other respects the two terminations are closely similar: open ocean forms do not survive and coastal forms flourish. We see small unspecialised planktonic foraminifera reminiscent of today's upwelling regions, and blooms of *Braarudosphaera* ('disaster forms' of Percival and Fischer¹³), as reported by Maxwell *et al.*⁶³ for the South Atlantic, and by Roth⁶⁴ for the Indian Ocean. In this view then the overall parallelism of plankton aspects of Palaeocene and Oligocene are due to the same cause.

The main difference between the two terminations (besides the amplitude) is the effect on benthic forms. At the Eocene–Oligocene boundary they are severely affected by the onset of production of cold bottom waters^{18,21,23}. We ascribe this difference between the Maastrichtian and the Eocene termination to a feedback effect, which had negligible consequences in the first, but greatly increased snow cover (and possibly ice formation) in the Antarctic during the second. Our argument is derived from first principles of physical oceanography and climatology⁶⁵. The introduction—presumably repeatedly—of a low salinity layer on top of the world ocean had the effect of temporarily obstructing the heat exchange between deep and shallow waters. As far as the heat budget of the Earth is concerned, such layering has the effect of reducing the ocean to a film of water hundreds of metres thick instead of thousands. Thus, the moderating influence of the ocean on climate is greatly reduced and seasonality increases correspondingly. The layering effect also increases the temperature of surface waters in the tropics, for the time of its duration. Both increased high latitude seasonality and increased tropical temperature favour transport of humidity to polar areas and its precipitation as snow. Once snow spreads, positive albedo feedback has a tendency to keep it there⁶⁶, resulting in a perennial polar high pressure cell with ensuing aridity.

In this view, then, the terminal Eocene injection event was the trigger for covering Antarctica with sufficient snow to move the polar front out to the shelf, which resulted in the production of cold bottom water. The cooling came at a time of regression, and albedo feedback through dry land areas⁶⁷ could be responsible for the overall drop in tropical temperatures in the Oligocene ocean⁶⁸. We do not doubt the importance of the circumpolar current in this context⁶⁹ but merely argue that it is part of the setting, together with the polar position of Antarctica⁷⁰, and that the opening of Drake Passage (the timing of which is very uncertain) was not the trigger event. The botanical evidence indicates that vascular plants persisted on the Antarctic coast into the late Oligocene and that complete coverage by ice did not occur before Miocene time^{71,72}.

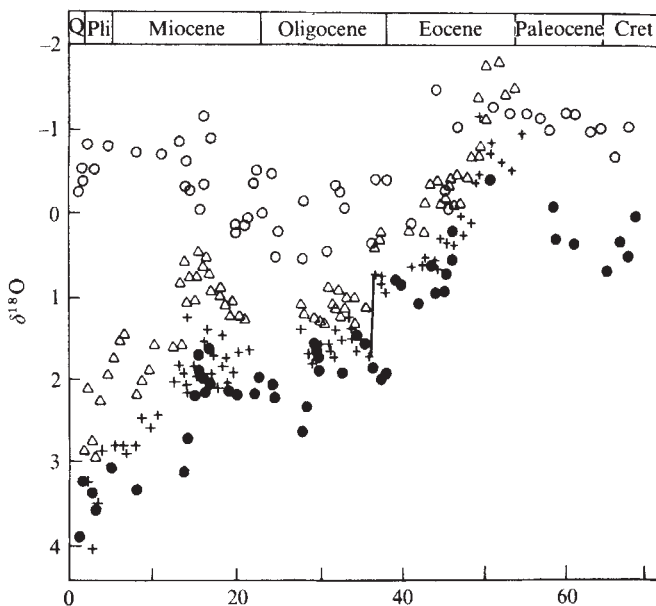
Messinian event

Isolation of the Mediterranean started in the early Miocene at its eastern end and culminated in the Messinian evaporite event^{33-36,73-75}. We note the great amount of salt estimated to have been deposited in the basin during the latest Miocene (6% of ocean total)³⁷. Many inundations were necessary to advect this salt⁷⁶ and intermittent water exchange with the world ocean probably occurred. In this view, the Mediterranean acted as a pulsating injector of saline waters, much like the South Atlantic some 100 Myr earlier. Again, stability of the world ocean was increased intermittently, with the described effect on seasonality, cooling and ice formation. An unusual cooling in high southern latitudes is shown for Site 284 (ref. 24) at the appropriate time, and the formation of ice strongly increased, according to the oxygen isotope curve from Site 281 (ref. 21). A temperature peak at the end of the Miocene is suggested for mid latitudes in the north-east Pacific⁷⁷. The co-occurrence of warming in the subtropics and cooling in high latitudes would agree with our hypothesis. We do not doubt the importance of the correct setting for increasing the formation of ice, including the closing of the Panama Passage^{78,79}, but stress the role of stratification as a trigger of positive-feedback mechanisms.

Oxygen isotope stratigraphy

If stable stratification is associated with the events under discussion, and if it has the effects postulated, we should see warming in low latitudes and increased seasonality in high latitudes. Unfortunately, our hypothesis cannot be tested using the available information. The following statements illustrate the type of analysis necessary for evaluating the reality of injection events, and their ramifications (Fig. 1): (1) in the published records there is no substantial change of oxygen isotope values between the late Cretaceous and Palaeocene, although a minor temperature fall across the Cretaceous–Tertiary boundary seems to be indicated^{80,81}. Thus, there is no evidence that the termination event acted as a trigger within a setting favouring change. Average temperatures were

Fig. 1 Oxygen isotope values of Cainozoic foraminifera (after Savin⁶⁸). Data mainly from Douglas and Savin⁸¹ and Shackleton and Kennett²⁵. The short straight line at the Eocene–Oligocene boundary indicates the position of the drastic isotopic temperature drop (see ref. 23). Central North Pacific: ○, planktonic; ●, benthic; Southern Ocean: △, planktonic; +, benthic.



apparently fairly similar before and after the event. (2) The oxygen isotope values for early Eocene planktonic foraminifera of Site 277, south of New Zealand are the same or lighter than those from Site 47 on Shatsky Rise then located in the subtropics. For a 5 °C difference, for instance, between subtropical and subantarctic temperatures these southern values are too low by at least 1‰. We ascribe this difference to evaporation-precipitation effects⁸², and postulate a difference in salinity of at least 4‰. Rainforests existed in Eocene time in southern Australia and in the Ross Sea area⁷². This supports our deduction from heat budget considerations of high rainfall in high latitudes. (3) The $\delta^{18}\text{O}$ values of low and high latitude foraminifera (and benthics) begin to separate in the mid-Eocene. This indicates that the global temperature gradient increased and supports our contention that the Eocene termination was a trigger event, accelerating the existing trend. (4) There is no indication of a sharp lowering of tropical temperatures at the very end of the Eocene. Our hypothesis would not favour such a sharp lowering (instead it calls for climatic instability). (5) The drastic mid-Miocene drop of high latitude isotope values, due to an unknown combination of making Antarctic ice²¹ and of further cooling⁶⁸, occurs concomitantly with high tropical temperatures. Although we do not postulate injection for this event, we see the marked transgression at the time^{83,84} as an event simulating injection of light surface water. The transgression leads to the formation of a warm water layer, by the additional capture of solar radiation on the flooded shelves.

We have not shown the available oxygen isotopes from the mid-Cretaceous as they are still too scarce for the type of analysis presented. Nannofossil data from the North Pacific⁸¹ show maximum temperatures near the Albian-Cenomanian transition, as predicted by our hypothesis. The same is apparently indicated by data from European belemnites, although various interpretations are possible⁸⁵.

Carbon isotope stratigraphy

The data for carbon isotope stratigraphy have been summarised by Fischer and Arthur⁶⁰. We agree with their conclusion that periods of strong oxygen minima correlate with the deposition of organic carbon which is enriched in ^{12}C (ref. 86) and with depletion of ^{12}C in carbonates^{12,83}. However, over short time-scales, we postulate that the oxygen minimum produced by an injection event will tend to enrich the plankton shells in ^{12}C (ref. 87).

In agreement with this expectation, we note marked enrichment with ^{12}C in the planktonic foraminifera just before the Eocene termination, in the data given by Shackleton and Kennett²¹. Also, a slight warming is indicated in the detailed oxygen isotopic curve of Kennett and Shackleton²³, just before the event, which again agrees with the concept of increased stratification as the trigger. Likewise, there is a remarkable enrichment in ^{12}C of *Uvigerina* from Site 284, at the very end of the Miocene²⁵. We interpret this to indicate stable stratification and oxygen depletion with concomitant increase of dissolved ^{12}C , from combustion of organic matter. The stable stratification, by our hypothesis, is provided through Mediterranean injection.

Preservation stratigraphy

Injection events should produce a preservation signal, as they would change mixing rates of the ocean, and hence fertility of the photic zone and affect the p_{CO_2} of deep waters. As in the interpretation of all other signals, caution is necessary to separate effects of a trigger event from those of the change in setting which ensued.

For the Cretaceous-Tertiary boundary widespread dissolution has been proposed^{12,88}. This idea is based on evidence from shallow sections, and may be correct for the upper water layers. In the sections at Caravaca (south-east Spain), Zumaya (Gulf of Biscay) and at Stevn's Clint (Denmark), for example, there is a dearth of carbonate in a thin boundary layer of

clayey sediment deposited in anaerobic conditions^{13,54,89-90}. A widespread oxygen minimum developing underneath a low salinity lid could produce such a signal. In the deep Atlantic, the transition is not marked by a dissolution event. In contrast, at Sites 356 and 384, for example, the foraminifera and nannoliths in the basal Tertiary (*G. eugubina* Zone) show excellent preservation^{52,91-93}. At site 358 there is a preservation climax in the lowermost Palaeocene⁹¹.

The Eocene-Oligocene event is marked by a sudden drop of the CCD from high, ridge crest elevation to abyssal depths^{19,94-96} and in the ratio of planktonic-benthic forams⁹⁷ which can be interpreted as indicating improved preservation, and also decreased productivity⁹⁸.

Another clue to the type of signal one might expect is given by the last deglaciation, 14,000-9,000 yr ago, when large amounts of meltwater were injected into the global ocean which apparently resulted in stable stratification of surface waters⁹⁹. This event was accompanied by a preservation spike which ended abruptly in a dissolution pulse¹⁰⁰. A simple explanation of this sequence would be that surface fertility was lowered during maximum stratification, letting the deep waters move towards chemical equilibrium, and that dissolution started when productivity resumed in upper waters and supplied organic carbon to still stagnating deep waters. However, the interpretation of the deglacial preservation sequence is complicated by re-deposition of shallow water sediments at the end of the glacial and by Holocene carbonate build-up on the flooded shelves¹⁰¹.

We believe that all three preservation spikes can be explained by a fertility crisis in the photic zone and ensuing saturation of bottom waters. In the case of the Maastrichtian-Danian boundary event excessively stable surface stratification may have prevented reflux of nutrients, and a widespread oxygen minimum might have destroyed available nitrogen¹⁰²⁻¹⁰³. The decay of stratification after the injection at the Eocene-Oligocene boundary was accelerated by the progressive replacement of haline circulation by thermal circulation⁸³.

For the mid-Cretaceous event, the evidence indicates a worldwide rapid shallowing of the CCD in the Aptian, a temporary lowering in the Albian-Cenomanian, followed by another dissolution pulse in the Turonian². The variation in carbonate deposition is closely matched by enrichments in organic carbon in deep-sea sediments, which parallels a general change from low oxygen content (carbonaceous sediment) to ventilation (oxidised sediment)⁶⁰.

The mid to late Miocene dissolution climax^{104,105} coincides with the beginning of isolation of the western Tethys, as well as a marked transgression^{83,84}. Of special interest is a dissolution pulse across the Miocene-Pliocene boundary, resulting in a high concentration of the genus *Sphaeroidinellopsis*^{91,106}. We believe this is due to an increase in deep-sea p_{CO_2} resulting from haline injection. This dissolution pulse is, incidentally, superimposed on a general trend of a falling CCD and improvement in preservation, showing that the terminal Messinian event does not produce the trend, although it may have accelerated the change at the boundary. In Core RC 12-66, from the east equatorial Pacific, maximum contrast of high and low carbonate values occurs at the end of Magnetic Epoch 5, near the boundary¹⁰⁷. The dissolution event in the earliest Pliocene seems to be the most severe one over several million years (event X in Kaneps¹⁰⁸). According to our hypothesis, this event in the Pacific and the *Sphaeroidinellopsis* zone in the Atlantic realm are both expressions of an increase in p_{CO_2} due to stable stratification.

The available information on isotopic stratigraphy and on preservation stratigraphy is compatible with our injection-stratification hypothesis. We now return to the major event of the past 100 Myr, the Cretaceous-Tertiary boundary, to test further the compatibility of hypothesis and observation.

Cretaceous termination at site 356

Recent successes in drilling the deep-sea contact of the Cretaceous-Tertiary boundary^{109,110} have made it possible to

study in detail this major event in ocean history¹⁴. Here we present the first detailed stable isotope stratigraphy across the boundary, closely dated with coccoliths. Procedures and supporting material will be reported in more detail by H.R.T. *et al.*

The oxygen isotope and carbon isotope determinations shown (Fig. 2) were done on the fine fraction (<62 μm). We believe the record is continuous or nearly so, because it correlates well with that from Site 384, which has also been dated biostratigraphically in great detail¹⁴. In both sections the evolution of early Danian assemblages is characterised by an initial bloom of *Thoracosphaera* spp., with *Markalius astroporus*, *Zygodiscus sigmoides* and *Cruciplacolithus* spp. present, and by a subsequent bloom of *Braarudosphaera* spp. and dominance of *Cruciplacolithus* spp. In both sections the latest Cretaceous and particularly the earliest Danian nannoliths are well preserved^{56,61}. It has been shown that in well preserved samples the stable isotopic variation of the nannofossil fraction parallels that of the planktonic foraminifera^{111,114}. At Site 356 the uppermost Cretaceous oxygen and carbon isotope values are near -2.0% and $+1.9\%$ respectively, which are typical for Maastrichtian plankton elsewhere in low latitudes⁸¹. At the termination event both $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ values drop sharply within 40 cm. 'Warming' and the formation of a strong oxygen minimum are apparently indicated. The Danian coccolith flora is dominated by thoracosphaerids and braarudosphaerids, neither of which, however, exceeds 25% of the total fine fraction in any sample.

How can the sudden warming across the Cretaceous-Tertiary boundary be produced? In principle, a large amount of heat could conceivably be supplied from space (for example, by a solar flare) or from Earth's interior (for example, large outpourings of submarine lava). We find both hypotheses unattractive, although the evidence may be insufficient to reject them outright. According to the injection hypothesis, the warming has a strong signal of fresh water composition in it, which makes it unnecessary to search for an extraneous heat source. Lowered salinity readily explains both the demise of the tropical late Cretaceous forms and the bloom of typically coastal braarudosphaerids. The magnitude of the fresh water signal can be estimated very roughly. Assuming that the precipitation of water in high latitudes in the latest Cretaceous was tagged with the isotopic signal of today's mid-latitudes in the rainbelt⁸² we obtain, say, $\delta^{18}\text{O} = -7\%$ for the fresh water injected from the Arctic. We take the average seawater $\delta^{18}\text{O}$ value as 0‰, for simplicity, and the salinity as 35‰. This yields a $\delta^{18}\text{O}$ of 0.2‰ for each 1‰ salinity change, when mixing fresh and saltwater. The

excursion of $\delta^{18}\text{O}$ towards low values at termination time is -2.0% , which translates into a lowering of salinity by 10‰, to 25‰, if the entire signal is due to mixing with fresh water. To the degree that warming occurs in the postulated stable surface layer, the salinity difference between normal and diluted seawater would be lessened. However, the difference must be increased to the degree that benthic mixing and the admixture of laterally transported Cretaceous nannoliths reduced the amplitude of the isotopic signal. We think that the estimate of a surface water salinity of 25‰ during the time of crisis is reasonable.

With the $\delta^{13}\text{C}$ signal, two effects must be considered. First, a marked decrease of plankton productivity would decrease the production of ^{12}C rich solids such as faecal pellets. The lack of removal of organics from surface waters would then decrease the carbon fractionation. Second, strong surficial stratification would result in the formation of a shallow, strong oxygen minimum. The effect of subsurface anaerobism on the $\delta^{13}\text{C}$ value in water can be estimated¹¹²⁻¹¹⁴. The relationship between the carbon isotopic composition of total dissolved carbon dioxide and the apparent oxygen utilisation (as calculated from Williams *et al.* (Fig. 2)¹¹³) is approximately $\Delta\delta^{13}\text{C} = -\text{AOU}/133$, where $\Delta\delta^{13}\text{C}$ is in ‰ PDB - 1 and AOU is in $\mu\text{mol l}^{-1}$. For an initial content of about 200 μmol of oxygen l^{-1} in warm surface waters of normal salinity¹¹⁵ and for total consumption, we get a signal of -1.5% in the $\delta^{13}\text{C}$, all other factors being equal. This signal is too small to explain the observed change which is also muted by the considerable reworking of Cretaceous taxa. The injection hypothesis allows for a higher initial oxygen content due to reduced salinity and cooler (Arctic) water, by about 20-30%. Thus, a signal of about -2.0% is justified under the hypothesis. In addition, the demonstrated deviation from equilibrium of calcareous nannoliths towards lower $^{13}\text{C}/^{12}\text{C}$ ratios¹¹⁴ may have been increased during post-termination time, due to sporadic bloom conditions, suggested by high abundances of thoracosphaerids and braarudosphaerids.

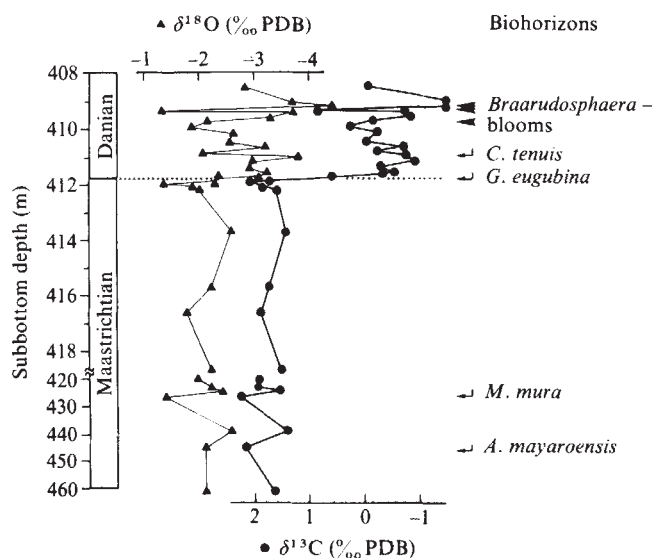
The rapid fluctuations in isotopic values in post-termination time seem to be real despite the somewhat wider sample spacing in the Upper Cretaceous. In part, such fluctuations can be produced by inhomogeneous benthic mixing, which has been observed at this boundary¹⁰⁹. However, mixing can only scramble the frequency, and decrease but never increase the amplitudes of the signal. Thus, the impression remains that large fluctuations characterised post-termination time. This is also indicated by the sporadic blooms of *Thoracosphaera* ssp. and *Braarudosphaera* ssp.

At least two mechanisms exist for producing the fluctuations in the framework of the injection hypothesis: (1) repeated isolation and opening of the Arctic which led to repeated injection of freshwater; (2) climatic oscillations involving alteration of strong cooling and warming. Climatic oscillations could come from the effects of intermittent stratification on the CO_2 exchange of the ocean and the atmosphere¹¹⁶. Such oscillations together with decreased climatic equability would also have a profound effect on the survival of land animals¹¹⁷. It is interesting, in this context, to contemplate the extinctions of large land animals at the last injection event, the deglaciation some 12,000 yr ago¹¹⁸.

We think that intermittent rapid addition to the world ocean of waters of unusual density produced in temporarily isolated basins in high and low latitude settings may account for some of the most puzzling oceanographic events discovered by deep-sea drilling, events which left their marks as mass-extinctions and widespread black shale deposits. Our hypothesis has implications for all Phanerozoic evolution, including the major mass extinction at the Permian-Triassic boundary.

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Fig. 2 Stable isotope curves of the fine fraction carbonate for DSDP Site 356, Sao Paulo Plateau, South Atlantic (analysis by John S. Killingley).



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Do present levels of air pollution outdoors affect respiratory health?

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A sensitive lung function test does not show differences due to air pollution between lifetime residents in a rural area and those in a small industrial town in Connecticut. Also, there is no evidence that higher air pollutant concentrations elsewhere have any marked effects on the lungs. Severe pollution is dangerous and must be avoided, but at present, air pollution control outdoors does not deserve priority as a means of preventing chronic lung diseases.

WE have assessed the impact of urban residence on respiratory health in the population of an industrial US town, in comparison with a rural population which included lifetime dwellers as well as migrants who had previously lived in cities. We determined differences in respiratory health between these populations from (1) the prevalence of common respiratory symptoms, and of bronchitis and asthma, and (2) lung function tests which reflect airway calibre. As air pollution outdoors is one 'urban factor' which may affect the health of people living in cities, we monitored several air pollutants at different sites in both towns.

The urban area had a record of high air pollution (by US standards) in the recent past, but the urban-rural pollution contrast at the time of our study was modest. We therefore also compared our results with data on people living in more severely polluted urban areas, and in other rural areas, so as to expand the pollution contrast.

To assess respiratory health in both towns, we used a questionnaire and a sensitive, standardised lung function test. We studied children and adults of both sexes, and healthy persons as well as those with respiratory symptoms or disease. Our population samples were large and representative of the total population of both areas. In analysing the results, we took into account sex, race, age, body height, body weight, smoking habits, occupation and previous residence. We believe that this is the first report of a study which combines all these features, and that it allows more reliable conclusions concerning urban factors and chronic respiratory disease (other than lung cancer) than do previous studies.

Populations and air pollution

The urban site, Ansonia, Connecticut, is an industrial town where mean annual particulate concentrations were among the highest measured in Connecticut during 1966-72 ($88-152 \mu\text{g m}^{-3}$; ref. 1); high SO_2 concentrations (by US standards) were probably also common in the past. In 1973, particulate concentrations were lower but still significantly higher than at the contrasting rural site, Lebanon, Connecticut. Lebanon is a sparsely populated town without factories or major highways, away from cities. Outdoor pollutants (Table 1) were monitored in both towns for more than 1 yr, including the period of our population surveys^{1,2}. Concentrations of total suspended particulates (TSP; high-volume samplers) and of nitrogen dioxide were significantly higher at the urban sites, as were nitrates and sulphates (data in refs 1, 2). There were no significant differences for sulphur dioxide and ozone.

In two geographically defined areas (the town of Lebanon and the 4th Ward of Ansonia), we attempted to study all residents aged ≥ 7 yr. Response rates varied from 91-96% among boys and girls (7-14 yr) in both towns to 56% and 80% among 25-64-yr-old adults in Ansonia and Lebanon, respectively. From a private census and interviews of nonresponders³, we concluded that the responders adequately represent the total populations. There were only 20 black residents of Lebanon; our main analyses are therefore limited to the white residents of Lebanon and Ansonia (Table 2). Of the Lebanon subjects, 41% had previously lived in urban areas; possible selection factors made it important to consider these subjects separately from lifetime rural (LR) dwellers. The few 'previous rural' (PR) Ansonia residents were excluded from analysis. Three smoking categories were considered: lifetime nonsmokers, ex-smokers of cigarettes, and current cigarette smokers (Table 2).

Questionnaire

We used an extended version of the MRC bronchitis questionnaire (for text, see ref. 4). Questions were prompted by computer and read by trained interviewers; answers were recorded in computer memory. There has thus been no omission or loss of data. Detailed written instruction and supervision of interviewers by the investigators promoted consistency in the interviews. We were unable to allocate subjects at random to interviewers, but an examination of symptom prevalences by interviewer showed that interviewer variation had no important effects on our results. Previous urban (PU) and lifetime rural residents of Lebanon were seen by the same interviewers; interviewer variation can be excluded as a cause of differences between these groups.

Chronic bronchitis

First, we examined the symptom complex of chronic bronchitis (usual cough and phlegm, more than 3 months per year for ≥ 2 yr). In groups of men and women by age (25+ yr) and smoking habits, smokers always had higher prevalences than

nonsmokers or ex-smokers, but urban-rural differences were absent in all groups. For example, 19.0% of the rural male adult current smokers had chronic bronchitis, compared with 17.0% of their urban counterparts. Among both urban and rural residents aged ≥ 45 yr, the prevalence of chronic bronchitis was less than half that found (with identical methods) among active and retired textile workers at risk from occupational cotton dust exposure⁵.

Asthma

A history of bronchial asthma ('yes' to the question: 'have you ever had bronchial asthma?') was more common among rural than among urban residents, regardless of age. The difference was highly significant for males (6.7% in 1,142 rural (=LR+PU) males; 2.6% in 458 urban males; $\chi^2 = 9.99$, $P < 0.01$) and similar differences, although not significant, persisted when only lifetime urban and rural residents were compared and when smoking was excluded by comparing only nonsmokers. Smoking habits were not significantly related to a history of bronchial asthma (χ^2 analysis).

Cough, phlegm and other symptoms

Chronic bronchitis may be an insensitive index of urban-rural differences because it is uncommon among nonsmokers. For its component symptoms, as well as wheezing and dyspnoea, we examined the relative importance of the residence variable and of sex, age and smoking habits in a weighted-least-squares analysis⁶ of all data in 15-64-yr-old nonsmokers and current smokers. We omitted children, because few of them had any symptoms, and the elderly, because they were too few in number. We also excluded ex-smokers; their symptom prevalences were usually close to those of nonsmokers. Table 3 summarises the best-fitting models. Cigarette smoking was the only variable consistently associated with increased prevalence of all symptoms, at $P < 0.001$. For usual cough and phlegm, a linear residence variable (that is, LU > PU > LR) was highly significant among nonsmokers but not among smokers. The linear residence variable for dyspnoea was complicated by interactions between sex and smoking and between LU residence and smoking. The association between residence and dyspnoea was most pronounced among nonsmoking women (that is, 12.8% in 211 LR compared with 19.2% in 151 LU women). Among nonsmokers (men and women), LR residents had the lowest dyspnoea prevalence; among smoking men and

Table 1 The two towns and their air quality

	Lebanon (Rural)	Ansonia (Urban)
No. of inhabitants	3,800	21,200
No. of inhabitants per km^2	29	1,178
No. of dwellings per km^2	12	248
No. of vehicles per km^2	26	650
No. of commercial buildings and factories per km^2	0.03	17.8
Sulphur dioxide ($\mu\text{g m}^{-3}$)	10.9 ± 1.6 (41)	13.5 ± 1.7 (50)
Total suspended particulates ($\mu\text{g m}^{-3}$)	39.5 ± 4.2 (44)	63.1 ± 3.7 (50)
Nitrogen dioxide ($\mu\text{g m}^{-3}$)	55.5 ± 6.1 (41)	87.8 ± 4.9 (50)
Ozone ($\mu\text{g m}^{-3}$)	84.7 ± 4.4 (28)	88.5 ± 3.3 (35)

Demographic and geographical data from 1970 US Census, US Geological Survey maps, and municipal registries. Air pollutant data were obtained at 3-5 sites in Lebanon and at 4 sites in Ansonia, from January to December 1973, and are means $\pm$ s.e.m. of 24-h average concentrations, except for ozone values, which are means $\pm$ s.e.m. of peak 1-h concentrations (no. of observations in parentheses). Pollutant measurements (for methods and details, see refs 1, 2) were made with supervised, accurately calibrated samplers placed 120 cm above ground level, at least 16 m from any road and at least 11 m from physical obstructions (such as homes, trees). TSP and NO_2 were significantly ($P < 0.001$) higher in Ansonia than in Lebanon; SO_2 and O_3 did not differ significantly.

Table 2 Population groups by sex, age, residence and smoking habits

Residence	Age (yr)					Smoking			Total
Males	7-14	15-24	25-44	45-64	65+	NS	XS	S	
Lifetime rural (LR)	232	116	152	96	18	342	122	150	614
Previous urban (PU)	117	49	135	72	20	165	90	138	393
Lifetime urban (LU)	81	81	71	74	17	144	77	103	324
Previous rural (PR)	2	6	9	10	5	14	9	9	32
Totals:	432	252	367	252	60	665	298	400	1,363
Females									
Lifetime rural (LR)	228	133	195	107	23	456	85	145	686
Previous urban (PU)	112	84	200	95	21	274	78	160	512
Lifetime urban (LU)	101	96	95	136	26	275	59	120	454
Previous rural (PR)	4	6	13	14	4	29	7	5	41
Totals:	445	319	503	352	74	1,034	229	430	1,693

LR, lifetime residence in Lebanon and other rural areas; PU, current Lebanon rural resident but past residence in urban areas; LU and PR, lifetime urban and previous rural residents of Ansonia. Total number of subjects (3,056) represents the total studied (3,387) minus 331 excluded for one or more of three reasons: (1) smokers of pipes or cigars only; (2) 1 yr or longer work in dusty occupations (mines, quarries, foundries, potteries, cotton mills, asbestos factories); (3) unclear history of previous residence (urban or rural). Lifetime nonsmokers (NS) had never smoked tobacco in any form; additional smoking of pipes or cigars by ex-smokers (XS) or current cigarette smokers (S) was not considered. Among children (7-14 yr) the number of ex-smokers and smokers was too small for analysis.

women the PU residents had the highest prevalence (30.8% compared with 21.5% in both LR and LU residents). Residence effects were less significant or absent for wheezing, and they were complicated by interactions with age. The significant age variables in Table 3 reflect higher prevalences among older than younger subjects, except for usual cough, where the 25-44-yr-olds had more symptoms than those who were younger or older. Sex was significant for usual phlegm, being more common among men, and for dyspnoea, which was more prevalent among women.

Figure 1 shows the observed prevalences of usual phlegm in relation to all variables which contribute significantly to its prevalence. There are no interactions, and the fit of the model is the best of all five in Table 3. Observed prevalences among women are similar to those predicted by the model. Among young urban male nonsmokers, the model seems to overpredict prevalence, but the difference between actual and predicted prevalence is not significant. All smokers, regardless of residence, have a higher prevalence of usual phlegm than do LU nonsmokers.

Lung function

We selected the maximum expiratory flow volume (MEFV) curve as a simple, sensitive and comprehensive lung function test⁴. It provides information on lung volume (forced expiratory vital capacity, FVC) as well as maximum expiratory flow rates. In previous studies, maximum flow at mid-vital capacity (MEF 50%) detected airway-constrictor effects of cigarette smoke⁷, textile dust⁸ and air pollutants⁹ better than did measurements such as peak flow or forced expiratory volume in 1 s (FEV_{1.0}). We recorded MEFV curves on-line with a computer¹⁰, calibrated every 2 h with a standard curve delivered by a mechanical device¹¹. From data on healthy lifetime nonsmokers (by race, sex and age group) in three US communities, including Lebanon and Ansonia, we have derived regression equations for FVC, FEV_{1.0}, the FEV_{1.0}/FVC ratio, peak expiratory flow rate (PEF) and maximum flow rates at two other points on the MEFV curve (MEF50% and MEF25%), as a function of age, height and weight¹². In the present study, these equations served to account for effects of age, height and weight in comparisons of lung

Table 3 Weighted least squares analysis of symptom prevalences

Symptom	Variables				Interactions		Fit of model		
	Smoking	Residence	Age	Sex	Age with:	Smoking with:	LU residence with:	P	% Variation explained
Cough	+++	+++*	++	0	Sex +++			0.719	74.8
Phlegm	+++	+++*	+	+++				0.998	81.1
Recent wheeze	+++	+†	+	0	Smoking ++		Age +	0.759	79.7
Frequent wheeze	+++	0	0	0	Residence ++			0.962	67.4
Dyspnoea 1+	+++	+++‡	++	+++		Sex ++	Smoking +++	0.910	77.7

The table includes variables and interactions which contributed significantly to the explanation of the variation in symptom prevalence among all lifetime nonsmokers (NS) and current smokers (S) in Lebanon and Ansonia (age 15-64 yr). Logistic models always gave a better fit than did additive models. If x is the proportion of subjects with symptoms, the logistic model uses a logarithmic transformation of x , that is, $\ln [x/(1-x)]$, as the sum of effects of smoking, residence, age, sex and their interaction. Definition of symptoms: Cough and phlegm on most days for at least 3 months per year; recent wheeze, wheeze within past 12 months; frequent wheeze, wheeze at least a few times each week; dyspnoea 1+, dyspnoea when hurrying on level ground or walking uphill, or worse. Significance of variables and interactions: +++, $P \leq 0.001$; ++, $0.001 < P \leq 0.01$; +, $0.01 < P \leq 0.05$; 0, not significant. Explanation of interactions: Age $\times$ sex: difference between sexes (M > F) increases with age. Age $\times$ smoking: age increase of prevalence greater for NS than S. Age $\times$ residence: 25-64-yr-old PU residents more wheeze than LR and LU; 15-24-yr-olds lower prevalence regardless of residence. Smoking $\times$ sex: less effect of smoking in females than males; NS females have relatively high prevalence. LU residence $\times$ age: effect of residence on prevalence increase more pronounced in 25-44-yr-olds than in 15-24- and 45-64-yr-olds. LU residence $\times$ smoking: LU smokers have lower prevalence than LR and PU smokers; LU nonsmokers have higher prevalence than LR and PU nonsmokers.

* Linear residence variable significant within NS only.

† LR < PU = LU, $P = 0.045$.

‡ Linear residence variable significant for NS and S.

function between groups of subjects. These comparisons were based on calculations of lung function residuals, that is, the differences between observed and predicted values of each measurement.

Analysis of variance of lung function residuals (Fig. 2) among LR, PU and LU residents by sex, age and smoking habits showed no significant differences for any of the measurements. LR, PU and LU nonsmokers had similar residuals, none of which differed significantly from zero or from each other. Smoking adults, on the other hand, had significantly more negative residuals than comparable nonsmokers, regardless of residence. Inclusion of adults with occupational exposure hazards (see legend to Table 1 for definitions) gave results similar to those in the population which excluded these persons.

Susceptible groups

Urban air pollution might not affect all residents, but only unusually sensitive groups within the urban population. Our data suggest that, if such groups exist, they cannot be readily identified on the basis of age (in those aged ≥ 7 yr), sex, race or smoking habits. Attempts to identify susceptible subgroups from questionnaire data were equally unsuccessful. Among smokers, the amount smoked seemed to be the main variable, affecting both symptom prevalence and lung function (unpublished observations), and there were too few nonsmoking urban (LU) residents who reported a history of asthma to allow a comparison of the severity of asthma among urban and rural residents. Among residents with usual cough and phlegm, lung function losses were minimal and not significant either among rural (LR) or urban (LU) residents. Thus, although there may be small groups of persons sensitive to factors in the urban environment that do not affect most people, we have not been able to identify them.

The 'urban factor'

Studies in several US cities¹³ have linked excess chronic bronchitis among smokers and nonsmokers to sulphur oxide and particulate pollution. In contrast, we have found that only lesser degrees of certain symptoms, not the composite syndrome of chronic bronchitis, may be associated with urban air pollution, and that this association only occurs in nonsmoking adults (age 15–64 yr; Table 3, Fig. 1). Among smokers, the influence of smoking overrides any differences associated with residence. However, the differences in symptom prevalences between urban and rural nonsmokers are not accompanied by differences in lung function. Even flows on MEFV curves (MEF50%; MEF25%), which are sensitive indices of airway obstruction¹⁴, did not differ. Thus, we have no objective evidence for substantial differences in respiratory health between urban and rural residents.

In the absence of lung function changes, what meaning should be attributed to the higher symptom prevalences among urban and previous urban nonsmokers? They might be due to trivial variables such as a different perception of questions by city and country people. The greater prevalence of dyspnoea among urban (LU) than rural (LR) nonsmoking women might be related to body weight (on average 4.1 kg more in 25–64-yr-old LU women than LR women). However, the LU-PU-LR gradient in prevalence of cough and phlegm among nonsmokers might reflect slight differences in respiratory health. For example, hypersecretion of bronchial mucus (leading to cough and phlegm production) may be more common among urban than rural nonsmokers. This disorder need not lead to progressive lung function loss¹⁵. It may be part of an adaptive mechanism (for example, increased mucus production may aid the clearance of pollutants) or it might represent incipient illness. Sputum from people in a polluted urban area may contain increased numbers of phagocytes and white cells¹⁶; these might secrete proteolytic enzymes which damage alveolar tissue¹⁷, or they may protect lung tissue against inhaled particles, or both. No firm conclusion is possible, but the lack of function loss among our older symptomatic, lifetime nonsmoking urbanites suggests

Table 4 Lung function in nonsmoking California and Connecticut residents

	Los Angeles	San Diego	Connecticut		
			LU	PU	LR
O ₂ ($\mu\text{g m}^{-3}$)	~300	~150	~100	~100	~100
TSP ($\mu\text{g m}^{-3}$)	124	78	65	42	42
No. subjects	90	135	73	41	56
Height (cm)	162	163	158	157	160
FEV _{1.0} (l)	2.44	2.43	2.10	2.08	2.25
MEF50% (l s ⁻¹)	3.38	3.37	2.96	2.81	3.09

Data for white, female, lifetime nonsmokers aged 45–64 yr. Los Angeles and San Diego data from Cohen *et al.*²⁸. Data for males are similar. Higher lung function values in nonsmoking California women are explained by their height. All function values are close to those predicted for healthy nonsmoking adult white females¹².

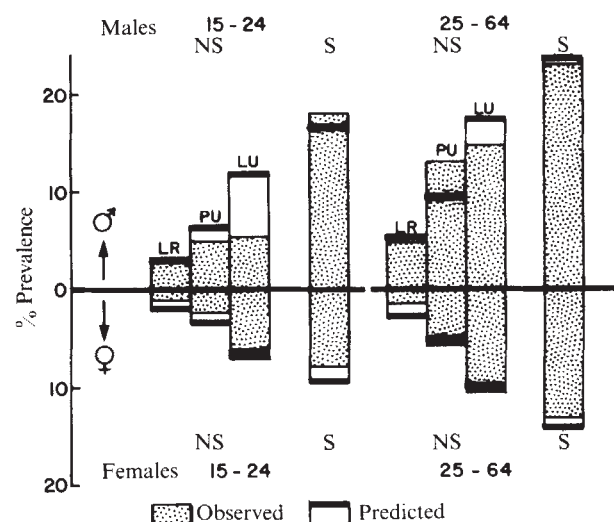
that the increased symptom prevalences are not associated with significant long-term deleterious effects on the lungs. Nor does our study indicate additive or synergistic effects between air pollution and smoking: lung function loss among smokers was similarly regardless of residence.

Our study cannot distinguish between air pollution and other urban factors as possible causes of the increased symptom prevalences among PU and LU nonsmokers. However, in a study designed to detect effects of air pollution peaks on daily symptoms among women in high- and low-pollution areas near Rotterdam, Wever¹⁸ found significant associations, mostly in nonsmokers, of pollution peaks with cough, phlegm and dyspnoea, but rarely with wheezing. The similarity of Wever's findings to our own supports the assumption that in Ansonia, too, air pollution may be the 'urban factor' responsible for increased symptoms in lifetime urban nonsmokers.

Expanding the pollution contrast

We have examined the effects of greater air pollution contrasts than existed between Ansonia and Lebanon in three ways: (1) by considering health effects of high indoor pollutant levels in homes, (2) by comparing our findings with those of others, in areas with pollutant concentrations higher than those in Ansonia, (3) by examining respiratory health data from people living in clean rural areas.

Fig. 1 Per cent prevalence of usual phlegm by sex, age, smoking and residence (LR, PU and LU explained in Table 2). Predicted prevalences are those obtained from the model summarised in Table 3. Only significant variables are shown; for example, smokers were not subdivided according to residence as this variable was only significant among nonsmokers.



(1) Suspended particulates indoors are predominantly small-sized ($<1\ \mu\text{m}$ diameter) and thus able to penetrate into small bronchi and alveoli¹⁹. Hence, with equal TSP concentrations, indoor air might be more damaging to health than outdoor air, in which large particles are kept suspended by air currents. However, we found no evidence that the high TSP levels (up to $400\ \mu\text{g m}^{-3}$) in homes with smokers (ref. 20 and H. R. Hosein *et al.*, unpublished observations) were associated with increased symptoms or lung function loss among nonsmokers in the same

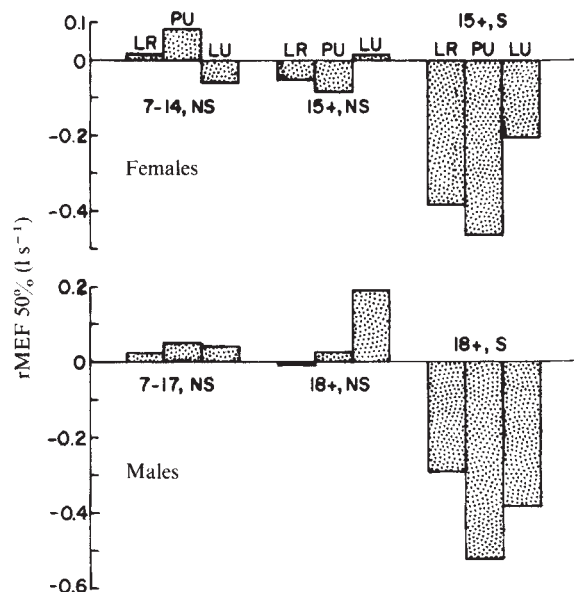


Fig. 2 Mean residual MEF50% (instantaneous maximum flow at 50% FVC). Residual (rMEF50%) = observed - predicted value. None of the differences between residential groups (LR, PU, LU, see Table 2) are significant in any of the six groups by sex, age and smoking habits. For boys and girls, this was also true when a further subdivision was made according to age (7-9 yr, 10-14 yr, 15-17 yr), and differences between LR, PU and LU residents were examined within each of these age and sex groupings. Differences between adult smokers and nonsmokers were significant within each residential subgroup (for example, LR smoking compared with nonsmoking males or females). For purposes of illustration, MEF50% is shown in l s^{-1} . The statistical analyses were, however, done on residuals in transformed units, $\ln \text{MEF50\%}$ in children and $\sqrt{\text{MEF50\%}}$ in adults; these transformations were required to obtain satisfactory prediction equations (see ref. 12 for the equations and their derivation). The equations with transformed units provide residuals which are normally distributed and have equal variances independent of age, height and weight within each subgroup by sex and age group. Values for s.e.m. residuals range over 0.037-0.085 in females and boys, and 0.1113-0.1755 in adult male groups. Differences between mean residuals in smoking groups may reflect differences in amounts smoked; although differences in amounts smoked were not significant between residential groups, PU men and women smoked more than LR and LU men and women.

homes. Nor did we find evidence for loss of lung function among children living in homes with parents who smoked²¹. Thus, much higher TSP concentrations than those which occurred outdoors in Ansonia may still be subthreshold with respect to health effects.

(2) SO_2 and TSP concentrations have previously been high in many urban areas. In the UK, very high pollution levels (up to $1,160\ \mu\text{g m}^{-3}$ SO_2) which occurred in 1965 and previous years, were clearly associated with exacerbations of bronchitis²². However, pollution levels lower than those in the UK in the 1950s and early 1960s do not seem to have any marked effect on health. For example, results similar to ours (a slight excess of cough and phlegm) were obtained among men employed in Manhattan in 1962-63 (ref. 23), before current air pollution control programmes began, and at a time when SO_2 and TSP

were of the order of $500\ \mu\text{g m}^{-3}$ and $250\ \mu\text{g m}^{-3}$, respectively¹³. At about the same time (1961-62), nonsmoking black and white male postal or transit workers in New York City^{24,25} had $\text{FEV}_{1.0}$ values almost identical to those in our urban and rural nonsmokers in 1973 when age, race and height are taken into account. According to our equations describing growth of lung function in children¹², Czech children (age 10-11 yr) living in a town with high SO_2 (annual mean $150-170\ \mu\text{g m}^{-3}$)²⁶ and TSP (annual mean $100-110\ \mu\text{g m}^{-3}$)²⁶ concentrations have lung function values²⁷ very close to those of 10-11-yr-old children in Ansonia and Lebanon.

Oxidant pollution has not been clearly linked with increased prevalence of chronic bronchitis or its component symptoms; in fact, chronic bronchitis seems to be about as uncommon among Los Angeles nonsmokers²⁸ as among our rural (LR+PU) nonsmokers of the same age (45-64 yr), that is, 1.7 and 1.5%, respectively. Nonsmokers in Los Angeles, exposed to higher oxidant and TSP concentrations, and nonsmokers in Connecticut have similar lung function when height is taken into account (Table 4).

(3) The lack of important contrasts in air pollution and respiratory health between Ansonia and Lebanon might be explained if Lebanon were a relatively polluted rural area. However, we have found no evidence that respiratory health is demonstrably better in pristine rural areas: Nonsmokers in such an area (Chilliwack, British Columbia) had higher prevalences of cough and phlegm²⁹ than our lifetime urban nonsmokers. Residents of Winnsboro, South Carolina (with significantly lower SO_2 and NO_2 concentrations than those of Lebanon¹) had symptom prevalences and lung function similar to those of Lebanon residents, and a higher prevalence of a history of asthma (unpublished observations). Black children and adults in a primitive village in Upper Volta, away from cities and with minimal automotive traffic³⁰, had FVC and $\text{FEV}_{1.0}$ values similar to those we recorded among urban black residents in Ansonia and rural black residents in South Carolina.

We conclude from these comparisons between our data and those of others that even relatively low levels of air pollution (as in Ansonia) may be associated with a slight excess of some respiratory symptoms in nonsmokers, but that these symptoms may only become noticeably worse when SO_2 and particulates reach levels higher than those prevailing in Manhattan in 1962-63. Even high concentrations of SO_2 , TSP and oxidants by current US standards are not associated with loss of lung function when sex, race, age, height and weight are adequately taken into account.

Implications

The overall impact of outdoor air pollution on respiratory health in Ansonia residents seems to be minimal. Even though the levels of air pollution in Ansonia are low by the standards of the UK in the 1950s, this is of interest because Connecticut is frequently said to have "the worst air pollution in the country outside Los Angeles" (ref. 31). However, any effect of air pollution in Ansonia is small compared with the effects of cigarette smoking and of certain occupational exposure hazards, such as occur among cotton textile workers⁵. Nor have we found evidence that outdoor air pollution worse than that in Ansonia (for example, in Los Angeles²⁸, New York^{23,24,25}, Rotterdam¹⁸ or Czechoslovakia^{26,27}) produces substantially greater effects on health. Our conclusions are valid for those lung disorders which affect vital capacity and air flow rates; these include asthma, bronchitis and emphysema. There may be sensitive subgroups within the general population which we have not been able to detect, but this remains speculation.

The extent to which communities want to abate air pollution, and the economical burdens which they are willing to accept for that purpose, are matters for society to decide. There are many reasons, aesthetic as well as hygiene-related, for reducing urban air pollution. Within rather wide margins, however, variations in air quality do not seem to have substantial effects on the

prevalence and severity of common diseases which affect airway calibre.

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Molecular structure of a double helical DNA fragment intercalator complex between deoxy CpG and a terpyridine platinum compound

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The crystal structure of a complex containing deoxy CpG and a terpyridine platinum compound (TPH) shows a DNA double helical fragment with TPH intercalated between two Watson–Crick GC base pairs. The DNA unwinding angle is 23° and the pucker of the deoxyribose rings differ at the 3' and 5' ends.

STUDY of the molecular structure of nucleic acid components is of considerable interest in developing an understanding of the macromolecular nucleic acids. Single crystal X-ray diffraction analysis allows us to determine unambiguously several geometrical details concerning these molecules. Studies from this laboratory^{1–4} showed that it was possible to crystallise self-complementary ribodinucleoside phosphates as double helical fragments with Watson–Crick hydrogen bonding between the bases. An interesting application of these studies is their extension to double helical fragments which crystallise with planar molecules intercalated between their base pairs. Lerman⁵ postulated that certain classes of planar molecules, many of which are mutagenic or carcinogenic, act by insertion between adjacent base pairs in the DNA double helix. Several structural studies have supported this interpretation. Sobell and his colleagues^{6,7} described the structure of an RNA double helical fragment which contained an intercalator and several different structures of this type have now been solved with a variety of

intercalators lodged inside double helical ribonucleotide fragments^{8–11}. These crystallographic studies show that the pucker of the ribose ring is modified by the insertion of an intercalator between the base pairs. However, a major difference between DNA and RNA double helices is the different pucker of the sugar ring. It is therefore of interest to ask how intercalation will modify the geometry of the DNA backbone especially with regard to the pucker of the deoxyribose ring¹².

In this article we report the crystal structure of a complex containing deoxycytidylyl-(3',5')-deoxyguanosine (deoxy CpG) which has crystallised with the intercalator 2-hydroxyethanethiolato-2,2',2''-terpyridine-platinum (II) [TPH]^{13,14}. In this structure, deoxy CpG forms an antiparallel double helix in which the helix has unwound and the base pairs are unstacked with one TPH molecule intercalated between the base pairs of the double helical fragment. Another TPH molecule is stacked between double helical fragments in the lattice. This structure allows us to determine directly both the unwinding angle of the DNA double helical fragment as well as the pucker of the deoxyribose rings.

Experimental methods

The dimer deoxy CpG was prepared using the phosphotriester method^{15,16}. The dimer thus obtained was converted into the ammonium salt by passing it twice over a Dowex cation-exchange resin in the NH₄⁺ form. The material was freeze dried

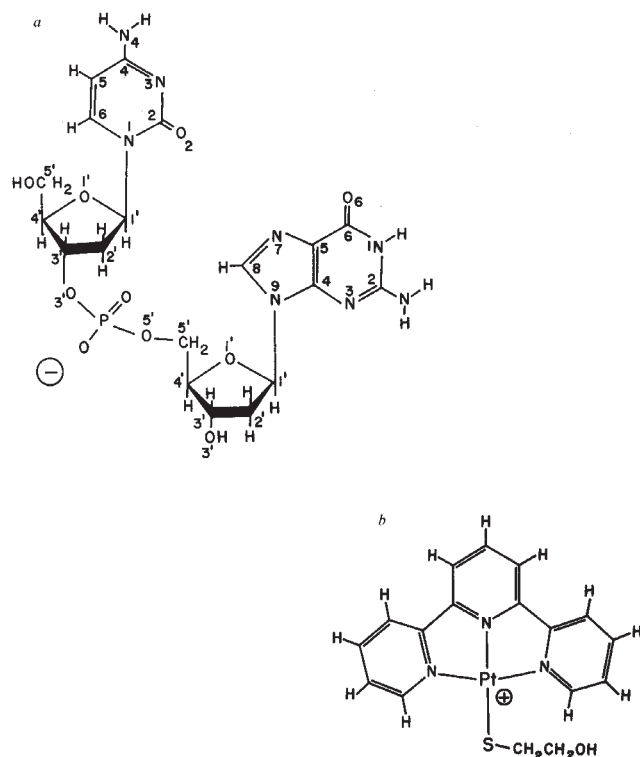


Fig. 1 Structural formulae of *a*, deoxycytidylyl-(3',5')-deoxyguanosine and *b*, TPH.

to a fluffy solid. The product was homogeneous and contained 3'-5' internucleotide linkages only (as shown using high pressure liquid chromatographic analysis and enzymatic digestion by spleen and venom phosphodiesterases). The intercalator TPH was prepared as described by Lippard and his colleagues^{13,14}.

Crystals were grown using the vapour diffusion method at room temperature in a manner similar to that used in crystallising tRNA¹⁷. An aliquot of 80 μ l of an aqueous solution containing 3mM NH_4^+ deoxy CpG, 3mM of the intercalator TPH nitrate, 25mM sodium cacodylate buffer (pH 6.0), and 17.5% 2-methyl-2,4-pentanediol was placed into a depression of a spot plate. The plate was sealed in a small chamber over a reservoir solution containing 75% 2-methyl-2,4-pentanediol. Orange-red plate crystals were obtained within 1 week. The UV spectra of dissolved crystals indicated a one-to-one stoichiometry of both the intercalator and the nucleotide. The material crystallised in the space group $\text{P}2_12_12_1$ with $a = 22.25 \text{ \AA}$, $b = 13.57 \text{ \AA}$, and $c = 33.12 \text{ \AA}$. Examination of precession photographs showed very strong reflections at 040 and 020 which correspond to planes at intervals of 3.4 and 6.8 $\text{\AA}$ along the b axis. This suggested stacking of base and intercalator planes perpendicular to the b axis which was verified by the structure determination. Data were collected at 8 $^\circ\text{C}$ with Ni-filtered $\text{CuK}\alpha$ X-ray radiation on a Picker FACS-I diffractometer to a resolution of 1.1 $\text{\AA}$. 3,519 independent reflections were measured with intensities greater than two standard deviations over their background. Data were corrected for Lorentz and polarisation factors as well as for absorption. A Patterson map revealed the position of two platinum peaks—one sharp and one moderately disordered. The structure was determined by difference Fourier ($F_o - F_c$) and sum function Fourier ($2F_o - F_c$) and was then refined by the least-squares method in successive cycles using a PDP 11-50 computer. The Nonius structure determination package was used. At the present stage of the refinement, the residual factor (R-factor) has been reduced to 15%. In addition to locating the intercalators and nucleotides, 20 water molecules have been identified in the asymmetric unit.

Results

Figure 1 shows the structural formulae of deoxy CpG and the intercalator TPH—singly charged molecules of opposite sign. The 2:2 complex is thus electrically neutral and no other ions are found in the lattice. Figure 2 shows a view of the molecule looking down the a axis in which the planar intercalator and base pairs are visualised virtually end on. In this view, it can be seen that the guanine and cytosine bases are paired together with three hydrogen bonds as is generally assumed to occur in DNA. The TPH in the centre of the diagram is intercalated between the two base pairs in the double helical fragment. The deoxyguanosine at the 3' end of the molecule has a deoxyribose with the C2' *endo* pucker, which is the normal pucker found in double helical B DNA¹⁸. However, the deoxycytidine at the 5' end has a modified pucker, namely C3' *endo* which is usually found in double helical RNA¹⁹ but is not found in normal B DNA. At the top and the bottom of the diagram are two intercalator molecules which are located at either end of the small double helical DNA fragment. These are related by a unit cell translation along the b axis, and are moderately disordered in the plane of the molecule. They are found principally in two locations, approximately 3.2 $\text{\AA}$ apart, located on either side of

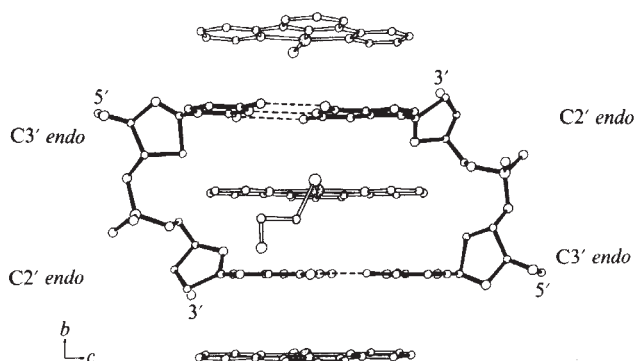


Fig. 2 View of the structure as seen down the a axis. The hydroxyethane side chains are not shown for the intercalators at either end of the double helical fragment. The glycosidic torsion angle (χ) is seen to be different at the two ends of the deoxy CpG. The two deoxyguanosine torsion angles are 114° and 117°; the angles for deoxycytidine are 32° and 34°. The phosphodiester linkage has a *gauche-gauche* conformation. Oxygen, sulphur, phosphorus and platinum atoms are drawn with larger circles than carbon atoms; the platinum atoms are stippled. Hydrogen bonds are drawn as dashed lines.

the pseudo-2-fold axis which is found parallel to the a axis at this level. The two sites occupied by this TPH molecule have nearly equal occupancies. Because of this disorder, the hydroxy-ethyl side chains of these stacking TPH molecules are not shown in Fig. 2. The complex has a non-crystallographic pseudo 2-fold axis passing through the intercalated TPH molecule parallel to the a axis. The hydroxy-ethyl side chain which is attached to the central intercalating TPH molecule effectively prevents the existence of an exact 2-fold axis at that point since the side chain does not have 2-fold symmetry as seen in Fig. 2.

Figure 3 is a diagram of the double helical DNA fragment containing the intercalating TPH molecule. This is a view perpendicular to the base pairs and the central intercalator. The difference in the pucker of the deoxyribose rings at the 5' and 3' ends of the molecule can be seen. It also shows the extensive overlapping of the π electron systems of the purine and pyrimidine rings with the terpyridine of the intercalator. Interestingly, all of the double helical ribodinucleoside phosphate intercalator structures which have been crystallised also contain the intercalator inserted in a pyrimidine-purine sequence⁶⁻¹¹. In

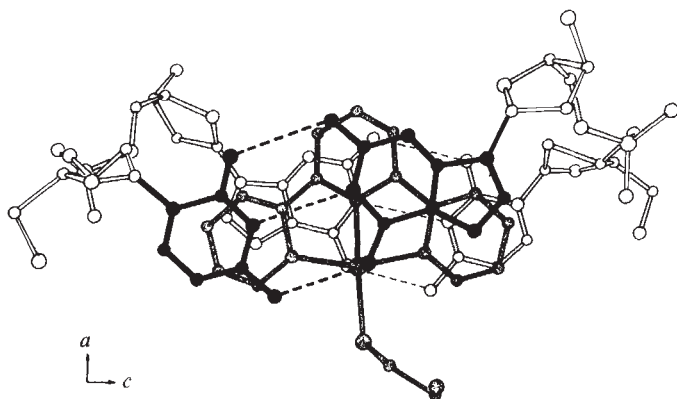


Fig. 3 A view of the structure down the *b* axis. Only the central intercalated TPH molecule is shown with the double helical fragment. The base pair closest to the reader is shaded solid black, the central intercalator is stippled while the bottom base pair is unshaded. The difference in deoxyribose pucker at the two ends of the chain is shown clearly here. Considerable overlap is evident between the bases and the intercalator pyridine rings. A pseudo-2-fold axis can be seen along the sulphur-platinum bond.

the native double helix there is less base stacking at the sequence pyrimidine-purine than at the sequence purine-pyrimidine. The stacking in the present pyrimidine-purine structure is considerably enhanced by the insertion of the intercalator, as seen in Fig. 3. For example, the outer pyridine rings are in a position where they provide a considerable degree of overlap for the purine ring on one side and the pyrimidine on the other. In a similar way the central pyridine ring of the intercalator overlaps the two purine residues in the centre of the molecule. Note that the oxygen O6 of both guanine molecules are located virtually above and below the central platinum atom in the intercalator, 3.4 Å away. It remains to be seen whether this feature plays a part in the interaction between this intercalator and the base pairs. It should be noted that the three unsaturated pyridine rings are organised in an arc which effectively overlaps the base pairs above and below. A similar arc-like arrangement is found in several of the unsaturated condensed ring intercalators such as ethidium and ellipticine⁶⁻⁹. As is shown in Figs 2 and 3, the side chain of the intercalating molecule is located in the major groove of the double helix. This orientation is in contrast to the TPH molecules which are stacked at either end of the double helical fragment. Here the side chain is located on the opposite side close to the narrow groove of the double helical fragment.

It can be seen in Fig. 3 that the TPH is intercalating in a symmetrical manner in this complex which is quite different from that which is seen in the ribonucleotide intercalation structures containing acridine orange, 9-aminoacridine and ellipticine^{9,11}.

The intercalator-double helical fragments are stabilised in the crystal lattice by a number of intermolecular interactions. These include hydrogen bonds from the hydroxyl group of the TPH intercalator to the cytosine O2 of another complex. There are also hydrogen bonds between the C5' OH of deoxycytidine to phosphate oxygens of an adjoining molecule which is related to it by twofold screw axes along the *c* axis. In addition, O3' of the deoxyguanosine residue is hydrogen bonded to O2 of the cytosine in an adjoining molecule. There are a number of water molecules which form both intramolecular and intermolecular hydrogen bonding bridges. An interesting intramolecular bridging hydrogen bonded water molecule is found connecting the C5' hydroxyl group and the phosphate oxygen of the same polynucleotide chain. This is responsible for the conformation of the C5' terminus of the oligonucleotide. However, this bridging water molecule is not likely to be present when the DNA is present in a normal double helical conformation.

It can be seen in Fig. 3 that the two GC Watson-Crick base pairs are related to each other by a right-handed helical rotation. In the B form of DNA the vector between the C1' atoms of the deoxyribose residues of the hydrogen bonded base pair are related to that of the next base pair by a rotation of 36°, as there are 10 base pairs per turn of the helix. In the present structure, however, the base pairs are no longer 3.4 Å apart but rather 6.8 Å apart due to the intercalator separating them. The inter-strand C1'-C1' vector is related to the corresponding vector on

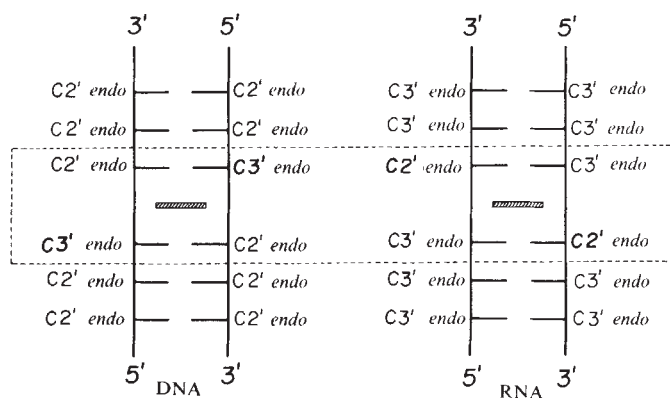


Fig. 4 A schematic diagram showing the sugar conformation in double helical DNA and RNA. The DNA is in the normal B form. The conformational effects of an intercalator are illustrated in the dashed region where the changes in sugar pucker are shown in bold face. For simple intercalators, the conformation in the dashed region is the same for the DNA and the RNA double helix.

the other side of the intercalator by an angle of 13°. Thus there is an unwinding of the double helix of 23° relative to that which it would normally have in the absence of the intercalator.

Discussion

The intercalator used in this study has several experimental advantages. The presence of the unsaturated terpyridine rings provide a substantial degree of overlap with the adjacent purines and pyrimidines. In addition, the centrally coordinated platinum atom significantly facilitates solution of the X-ray diffraction problem. This intercalator has been used extensively by Lippard and his colleagues in a variety of studies as reviewed recently²⁰. Intercalators of this type compete with ethidium and induce viscometric and spectroscopic changes which can be readily interpreted as due to interaction with DNA in an intercalative mode.

This crystal structure shows the manner in which the DNA double helical fragment accommodates the interposition of a planar molecule between the base pairs. In view of the extensive previous work with intercalators in fragments of an RNA double helix (Table 1), we can make a comparative study. The important difference between the DNA and the RNA double helix is the presence of the additional 2' OH group in the ribose of RNA, which makes RNA adopt a C3' *endo* ring pucker while in DNA the deoxyribose is normally C2' *endo*^{18,19}. This nomenclature refers to the displacement of either the C2' or C3' carbon atom out of the plane of the ring in a direction towards the base or the major groove of the double helix. One of the features of several RNA double helix-intercalator structures (Table 1) has been the observation that puckering of the ribose rings differs at the 3' and 5' ends of the intercalated segment. This is especially true with 'simple' intercalators which do not form hydrogen bonds with the nucleotide backbone. The same phenomenon is seen in the present structure. The DNA fragment shows a C3'

Table 1 Intercalation geometry of nucleic acid structures*

	Dinucleoside phosphate	Intercalator	Sugar pucker		Twist angle	Ref.
			5' end	3' end		
DNA fragments	dCpG	TPH ⁺	C3' <i>endo</i>	C2' <i>endo</i>	13°	This work
RNA fragments	rIodoUpA	Ethidium ⁺	C3' <i>endo</i>	C2' <i>endo</i>	8°	6, 7
	rIodoCpG	Ethidium ⁺	C3' <i>endo</i>	C2' <i>endo</i>	8°	8
	rIodoCpG	9-NH ₂ -Acridine ⁺	C3' <i>endo</i>	C2' <i>endo</i>	8°	9
	rIodoCpG	Ellipticine ⁺	C3' <i>endo</i>	C2' <i>endo</i>	10°	11
	rIodoCpG	Acridine Orange ⁺	C3' <i>endo</i>	C2' <i>endo</i>	10°	11
	rIodoCpG	Proflavine ⁺	C3' <i>endo</i>	C3' <i>endo</i>	36°	11
	rCpG	Proflavine ⁺	C3' <i>endo</i>	C3' <i>endo</i>	33°	10

* Two different classes of intercalators are indicated by the dashed line.

endo conformation at the 5' end of the dinucleoside phosphate and the normal C2' *endo* conformation at the 3' end, that is C3' *endo* (3', 5') C2' *endo* mixed sugar puckering. In the simple intercalated RNA helical fragments it is the 3' end of the ribonucleotide chain which is perturbed to adopt the C2' *endo* conformation which is normally found in the B form of DNA but not normally found in the double helical RNA chain. There are two interesting exceptions which occur with proflavine intercalation as shown in Table 1. These differences are likely to be associated with the fact that the amino groups on proflavine form hydrogen bonds with the phosphate group of the internucleotide link. This may have the effect of constraining the pucker which the ribose residues can adopt.

For the class of 'simple' intercalators which intercalate but do not hydrogen bond with the phosphate groups we see an interesting similarity in the manner in which the DNA double helix and the RNA double helix accommodate the intercalation (Fig. 4). Away from the intercalated site, the sugars of the two types of double helices have the different puckers mentioned above. At the intercalator site (Fig. 4) both polynucleotide chains have a similar C3' *endo* (3', 5') C2' *endo* conformation. This result supports the use of intercalated RNA helical fragments as models for studying the geometry of DNA intercalation. Such a mixed puckering conformation has also been suggested to occur in B DNA by a model building study²¹. This identity of conformation in the region surrounding the intercalators is due to a change in the conformation of the DNA segment at the 5' end of the internucleotide linkage surrounding the intercalator moving towards the conformation found in the RNA double helix. In the RNA double helix, a new conformation is adopted at the 3' end, the C2' *endo*, which is normally associated with the DNA double helix. The net effect of both of these changes is that it gives rise to the familiar nearest-neighbour exclusion effect which is found with intercalation. When double helical DNA or RNA is fully saturated with intercalators, only every other site can be occupied. This is because the conformational change adopted by the polynucleotide chain in accommodating the intercalator differs at the 3' and 5' end. One cannot obtain this conformation between every base pair but at best between every other base pair. An X-ray diffraction study of DNA fibres using the same terpyridine platinum complex used in this study demonstrated the nearest-neighbour exclusion in DNA fibres²². The interpretation was facilitated by the presence of platinum in the intercalator which scatters X rays strongly.

One of the features associated with intercalation in a DNA double helix is the fact that it gradually unwinds the helix. Studies of ethidium intercalation into supercoiled DNA has made possible a calculation of the unwinding angle in DNA associated with each molecule of ethidium²³. The studies indicate that insertion of ethidium between the base pair decreases the twist angle between the base pairs by $26^\circ \pm 2^\circ$. This value is very close to the 23° observed in the present structure and is also similar to the results seen with the insertion of simple intercalators into ribonucleotide fragments. In contrast, the

intercalator proflavine which hydrogen bonds to the phosphate groups has an unwinding angle of 0° .

In the present crystal structure one of the TPH molecules is used to maintain continuity of stacking between one double helical fragment and the next. That molecule is disordered in an interesting manner. In one of the two disordered positions, there is a maximum degree of overlap with the guanine residue in the double helical fragment below it. In the other disordered position, there is a greater degree of overlap with a guanine residue from the double helical fragment above it. One element in the disordering process may be due to the presence of two positions with similar energy minima. There is no disorder in the intercalated TPH molecule.

Single crystal analyses of nucleic acid fragments have considerable value in providing us with fine details associated with the manner in which these molecules adapt to a variety of environments. The present investigation has illustrated the manner in which the DNA backbone accommodates the insertion of an intercalator between base pairs. Studies similar to these, and especially those involving longer deoxyoligonucleotides should prove of great value in enhancing our understanding of the mode of action of DNA in biological systems.

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letters

Pulsed and unpulsed light from the Vela and Crab pulsars

OBSERVATIONS of the Vela and Crab pulsars were made with the Image Photon Counting System¹ (IPCS) at the $f/15$ focus of the 3.9 m Anglo-Australian Telescope (AAT), on 9 and 10 March 1978. Observations of the unpulsed light from the Crab pulsar have been obtained with an electronographic camera⁵ used with the 0.9 m Yapp reflector of the Royal Greenwich Observatory (RGO). We obtained phase-resolved pictures of the Vela pulsar^{2,3}, positively identifying it with the faint, blue candidate star⁴ and detecting a relatively strong, unpulsed light component. We also detected a weak unpulsed component from the Crab pulsar.

In the AAT observations the pulsar period was divided into eight phase intervals, and a picture was accumulated of the area around the pulsar during each phase interval. The eight pictures, each of 42×42 pixels (corresponding to 12 arc s on a side), were held in the memory of an Interdata-70 computer. Every millisecond, an interrupt from the observatory timing system caused the computer to calculate the current phase of the pulsar, and select to which of the eight pictures the next TV frame from the IPCS would be delivered. In this way, TV frames from the IPCS, generated every 6.2 ms, were stored and each picture in the computer memory became the integration of many frames from the same phase interval. Total integration times on the Vela pulsar were respectively 5 h 16 min (B) and 3 h 30 min (U). Crab pulsar observations were for ~ 10 min each with the telescope stopped down to 1.8 m aperture (central obstruction of secondary 1.4 m); this was to prevent saturation of the IPCS during the bright portion of the pulse. The Vela pulsar observations were all phased with respect to the dedispersed radio pulse arrival time, 21 ms before the first optical pulse. Predictions of radio pulse phase were made from radio measurements made as part of a continuing series of pulsar timing observations⁶. The Crab pulsar observations were obtained at arbitrary phases.

During the observations, the eight phase-resolved pictures were cyclically displayed on a TV monitor, so that the pulsar pulses were seen in stroboscopic fashion. Photographs of the display are shown in Fig. 1 for observations in blue light (B-band) for the Vela and Crab pulsars. Figure 1 confirms the identification³ of the Vela pulsar with the star known⁴ as M and clearly shows residual emission in its light curve outside the two main peaks, which fall in phases 2 and 4.

The intensities of stars in each field observed with the IPCS were determined by a least squares procedure which simultaneously fitted the sky background (three parameters), the positions and intensities of the stars (three parameters per star), and the stars' profile expressed as a two-dimensional semi-empirical point-spread function with central core and extended wings (five parameters). Nebulosity in both the Crab and Vela pulsar fields could produce systematic errors in the measurements of the pulsar intensities, and so was determined from the data by an iterative technique. The residuals resulting from an initial fit were smoothed by a broad spatial filter to determine the background variations whose scale was large, compared to the size of a star, but which was small compared to the size of the

field. This picture, representing the background nebulosity, was subtracted from the picture of the pulsar field, and the star intensities were obtained by reapplying our fitting procedure. The intensities of the Crab and Vela pulsars in the B and U filter bands are given as a function of pulse phase in Fig. 2.

A surprising result from the observations is the intensity of the emission from the Vela pulsar outside the two main peaks. The unpulsed emission accounts for about half the total emission from the pulsar. To estimate the intensity of the unpulsed light we have fitted higher resolution light curves to the data in the following way.

Each of the pictures within a set of eight covers an interval of pulsar phase. The edges of this interval are blurred by the IPCS response time and by the millisecond timing interrupts. Thus the pulsar intensities derived from those pictures which give the lowest intensities may include pulsed light from adjacent phase intervals in which the pulsars were brighter. This problem is severe in the case of the Crab (period 33 ms, phase interval 4 ms) but not as severe for Vela (period 89 ms, phase interval 11 ms). Light curves obtained in 1977 with an unfiltered S20 photomultiplier and high time resolution³ have been convolved with the appropriate smoothing function representing the instrumental smearing. Assuming that the shape of the light curve is unchanged in the past year and that the shape of the pulsed component does not depend much on the colour in which it is observed, we fitted the amplitude and baseline of the convolved light curve by a least squares procedure to the pulsar intensity derived from the IPCS observations. The fit of the convolved light curves to the observations is shown in Fig. 2a. The high time resolution light curves used for the convolution are plotted in Fig. 2b; for each curve the true zero is represented by the base of the plot.

The apparent magnitudes in U and B of the time averaged

Table 1 Pulsar magnitudes

	Crab	Vela
Mean apparent magnitude (B)	—	23.56 ± 0.03
Mean pulsed apparent magnitude (B)	17.0*	24.4
Mean steady apparent magnitude (B)	18.6	24.3
Mean apparent magnitude (U)	—	23.20 ± 0.14
Mean pulsed apparent magnitude (U)	16.5*	23.8
Mean steady apparent magnitude (U)	18.1	24.2
Distance ¹¹ (pc)	2,000	500
Distance modulus ($m-M$)	11.5	8.5
Absorption ^{3,12} (A_B)	2.1	0.5
Absorption ^{3,12} (A_U)	2.8	0.7
Mean steady absolute magnitude (M_B)	5.0	15.3
Mean steady absolute magnitude (M_U)	3.8	15.0
Ratio of steady/pulsed light expressed as a difference in magnitude (B)	1.6	-0.1
Ratio of steady/pulsed light expressed as a difference in magnitude (U)	1.5	0.4

* Ref. 10.

intensity from the Vela pulsar are given in Table 1. Calibration of the blue sensitivity of the IPCS was with respect to faint standard stars in the globular cluster ω Centauri (R. Cannon, personal communication), and the standard error quoted for the blue magnitude represents the internal consistency of the data, as the calibration is relatively well assured. Calibration of the UV sensitivity of the IPCS was with respect to star K (ref. 3) and with respect to UV measurements⁷ of brighter stars in ω Cen (no faint U standards are available). The brighter stars were observed with the telescope out of focus in an attempt to avoid

saturation problems of the IPCS. Because this calibration is less assured, a larger standard error for the U measurement is given based on the agreement within the methods.

Table 1 also gives estimates of the magnitudes of the pulsed and unpulsed light from each pulsar. The model chosen to distinguish pulsed and unpulsed light is that the high-resolution light curves representing the pulsed emission are supported on a steady pedestal of constant emission.

The presence of the small unpulsed background from the Crab pulsar, which amounts to $3.6 \pm 0.7\%$ of the peak intensity

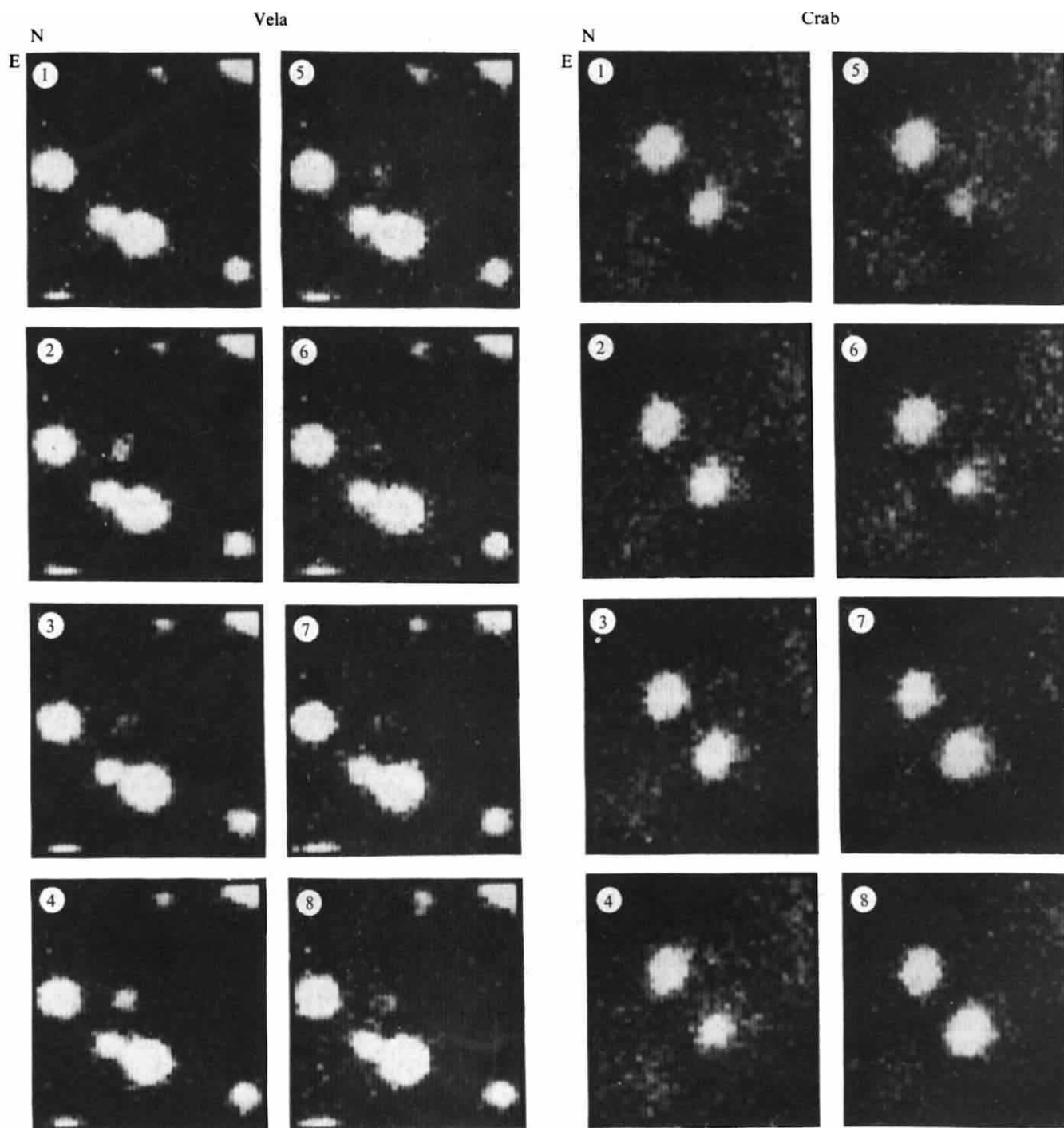
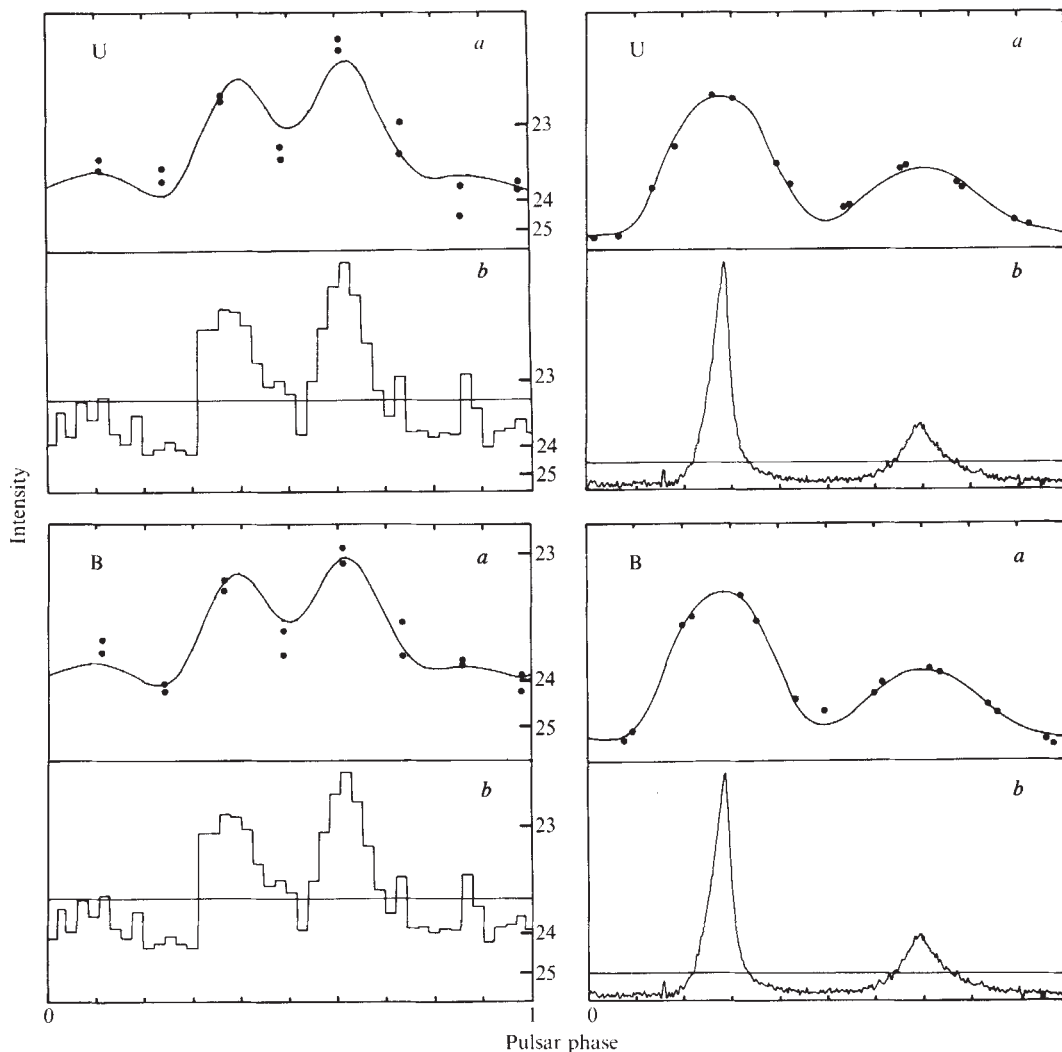


Fig. 1 The fields of the Crab and Vela pulsars in blue light as shown by the observing display at the Anglo-Australian Telescope. Each field covers an area on the sky 12×12 arcs, and one eighth of the phase of the pulsar's cycle. The fields are numbered in sequential phase order for each pulsar. The main pulse of the Crab pulsar occurs in phases 7 and 8, while the inter-pulse occurs in phases 2 and 3. The main double pulse of the Vela pulsar occurs in phases 2 and 4. Emission can be seen throughout the whole cycle of the Vela pulsar. (Emission seen throughout the whole cycle of the Crab predominantly results from instrumental smearing.)

Fig. 2 Light curves of the Crab and Vela pulsars in UV (U) and blue (B) light. Points represent the intensity of the pulsars as observed with the IPCS. (The data were divided into halves of equal weight to generate two points per phase interval.) As described in the text, curves were fitted to the data points by smoothing high-time-resolution profiles obtained with unfiltered S20 photomultipliers in earlier observations². The high-time-resolution profiles are shown in the lower panels (b) with their mean intensity indicated by the horizontal line. In all eight panels of this figure, the ordinate represents light intensity correctly zeroed at the base of each panel. Calibration to magnitudes is given on the right hand ordinate, for the Vela pulsar. The abscissa of each panel covers one cycle of the pulsars.



(but a surprisingly large 25% of the mean pulsed light) is not in serious disagreement with the estimated upper limit of 2% of maximum intensity given by Miller and Wampler⁸ on the basis of estimates from a TV picture taken through a strobing shutter with effective phase resolution of 20% of the pulsar period.

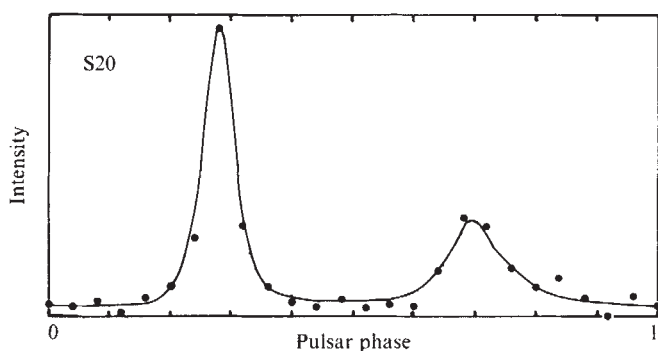


Fig. 3 Points are electronograph measurements of intensity of Crab pulsar summed in 25 equal sized phase bins: curve is high-time-resolution light-curve smoothed to 1.33 ms resolution and adjusted in amplitude, phase and base level to fit data. The minimum point of the curve (preceding the main pulse) is significantly above the zero level.

The unpulsed background of the Crab pulsar which we observe with the IPCS is confirmed by an independent observation which was also independently reduced. In this observation (made on 7 November 1977) the photocathode of a McMullan electronographic camera⁵ was masked to leave only a small area exposed in the focal plane of the RGO 0.9 m telescope (no filter, S20 photocathode). The picture area was stepped by deflection coils in synchronism with the pulsar period, to give 25 separate pictures, one of each phase bin. The response-time of the electronograph to a step of the deflection coils was very small compared with the 1.33 ms dwell-time for a picture. Using a PDS microdensitometer, each electronograph picture was scanned along the line joining the pulsar and its companion to the north-east, and the resulting profile across the pair of images was fitted with two one-dimensional point-spread functions, with similar form to the two-dimensional point-spread function used in the IPCS analysis. The ratio of the heights of the profiles is a measure of the intensity of the pulsar, because the electronograph produces an image whose density is linearly proportional to image intensity. We show the Crab pulsar light curve derived from an observation of duration 10 min in Fig. 3. The unpulsed background of the Crab pulsar detected with the unfiltered S20 electronograph is $3.7 \pm 0.8\%$ of its peak intensity, in excellent agreement with the IPCS observations in U and B.

The ratio of steady/pulsed light for the Crab pulsar is similar in B, U (Table 1) and S20 wavebands. However, for the Vela pulsar the steady component may be weaker in the UV band than in the blue band. We used the χ^2 -test to measure how

similar the two light curves were (to within a scale factor). The probability of the value of χ^2 which measured the goodness of the match between the two observed curves was 4% ($\chi^2 = 14.9$, 7 d.f.). A better match between the U and B light-curves occurred when an extra steady intensity, equal to 0.26 of the mean U intensity, was added to the U light curve ($\chi^2_{\min} = 10.0$, probability 20%). Thus the steady light component of the Vela pulsar may be redder than the pulsed component.

For both pulsars the absolute magnitude of the steady, unpulsed component (Table 1) is too great to be attributed to thermal emission from the neutron star from which the pulsed radiation is beamed. The thermal radiation from a neutron star 9 km in radius was calculated⁹ to have absolute magnitude $M_B = 19.4$ if the surface has a temperature $T = 2 \times 10^6$ K and would be $M_B \sim 25$ at $T = 10^4$ K.

There are several possible origins for the steady, unpulsed light component. (1) It may be pulsed light emitted from points distributed in space. Such light would reach the Earth with various arrival times and could contribute light at all phases. (2) A pseudo-steady component will also be seen if the pulses are so broad that they effectively fill the entire period. The high time resolution³ light curve of the Vela pulsar, (Fig. 2b) hints at two weak pulses, similar to the two main pulses, but 180° offset in phase. The two main pulses may be produced by one pole of the rotating neutron star while the two weaker pulses are produced by the other pole. (3) The steady component may be thermal or other radiation from material near the pulsar (such as, a nebular wisp) that is reflecting or reprocessing energy in the pulses. Alternatively, if the beamed emission originates away from the neutron star, at the speed-of-light cylinder for example, the steady emission may be this radiation reflected from the surface of the neutron star.

The indication in Table 1 that the steady component of the Vela pulsar may be redder than the pulsed component, implies that reprocessing may be occurring, although it remains to be explained why almost as much light is received at Earth as a result of reprocessing as is received at Earth directly from the pulsar beam.

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IUE observations of Large Magellanic Cloud members and detection of the 2,200 Å feature

THE dominant feature in the UV spectra of reddened stars in our Galaxy is the broad absorption feature of interstellar origin, centred near 2,200 Å and having a halfwidth of 350 Å (ref. 1). The wavelength position of this feature and the correlation of its strength with other interstellar extinction parameters, for example, the visual colour excess $E(B-V)$ are remarkably uniform throughout the Galaxy within 2 kpc from the Sun². The 2,200 Å feature is attributed to small graphite spheres with radii between 100 and 200 Å (ref. 3), but a molecular origin cannot be ruled out⁴. It is, therefore, the most important feature to search for in the interstellar extinction laws of other galaxies. The Magellanic Clouds are the nearest extragalactic systems and the Large Magellanic Cloud (LMC), in particular the region near 30 Doradus, contains a significant amount of dust⁵. In the past few years considerable doubt has been expressed about the existence of the 2,200 Å feature in the LMC extinction law as derived from surface brightness measurements of the region around 30 Doradus⁶, the results being rather inconclusive due to the difficulties arising from uncertainties in the luminosity function for the region concerned⁷. The interstellar extinction law can be reliably determined only from studies of the individual spectra of reddened and unreddened stars of similar spectral types. We present here the UV observations of a moderately reddened and a slightly reddened early type supergiant in the LMC obtained with the IUE. These observations answer the question of whether or not the agents responsible for the 2,200 Å feature are present in interstellar dust in the LMC.

The observations were obtained with the IUE at the low resolution mode ($\Delta\lambda \sim 6$ Å) over the entire wavelength range 3,200-1,150 Å. The spacecraft, its instrumentation, and the performance of the telescope, spectrograph and the SEC cameras are described elsewhere^{8,9}. The reddened star is Sanduleak no. SK-69-108 (ref. 10). This star ($V = 12.10$, $B-V = 0.27$) is classified as B3I (ref. 11) and has a visual colour excess $E(B-V) \sim 0.45$. The comparison star, SK-67-108, is situated in a region free from nebulosity. It is classified as B0I (ref. 11) and its reddening, estimated to be $E(B-V) \sim 0.05$ arises mainly in our Galaxy.

Fig. 1 Observed flux distribution of SK-69-108 (a) and SK-67-108 (b). The points indicated by + signs are due to a fiducial mark.

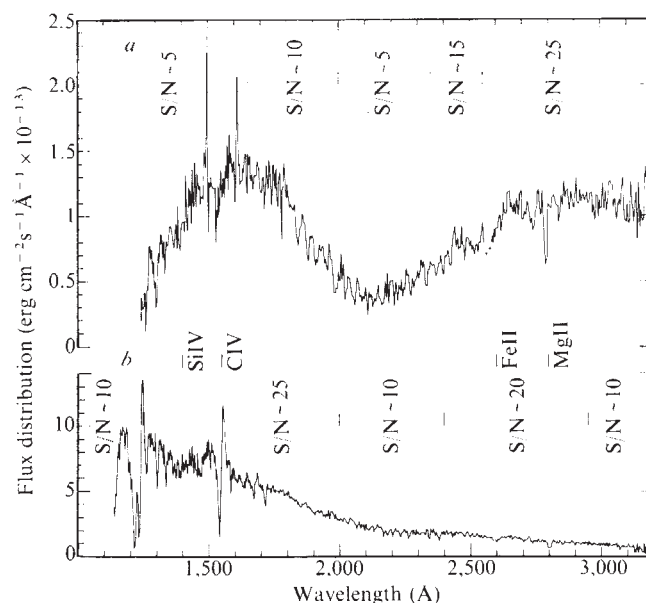


Table 1 Observations

Object	Species type	U	B	V	Image	Aperture	Exposure time (min)
SK-69-108	B3I	11.88	12.37	12.10	LWR1726	Large	45
					LWR1727	Large	90
					SWP1853	Large	80
SK-67-108	B0I	11.31	12.36	12.56	LWR1594	Small	20
					SWP1690	Small	20

LWR = long-wavelength redundant camera; SWP = short-wavelength prime camera.

The reddened star, SK-69-108, was observed with the large entrance slot (15×20 arc s), but the comparison star, SK-67-108 was observed with the small entrance slot (3×3 arc s). The photometric data, exposure times and image numbers are given in Table 1. The reduced spectra were provided by the IUE ESA observatory at Madrid, using the procedures described by Boggess *et al.*⁹. The wavelengths were checked using the positions of the major lines identified in the spectra. The raw image of each exposure was examined for saturated pixels, and only the unsaturated portions of the spectra were used. The net spectrum (observed minus smoothed background) was converted to absolute flux units using the preliminary system sensitivity functions provided by the Appleton laboratory.

Figure 1 shows the spectra of the reddened star SK-69-108 and the comparison star SK-67-108. The two peaks in excess of 20 units near 1,600 and 1,500 Å in the spectrum of SK-69-108 are due to charged particles incident on the camera; this is clear on the raw image. The mean signal-to-noise ratios computed from the counts recorded in the individual pixels of the raw image for different parts of the spectrum of each star are indicated in Fig. 1.

The C IV doublet at 1,550 Å in the spectrum of the unreddened star is very strong and shows a P-Cygni profile; on the other hand, the S IV doublet at 1,400 Å which, in early-type supergiants is usually as strong as C IV lines¹², is rather weak. Similarly, in the reddened star, the C IV doublet is considerably stronger than the S IV doublet. The most prominent line longwards of 2,500 Å is the Mg II doublet at 2,800 Å, and is present in both spectra. The line near 2,600 Å due to Fe II which appears clearly in the spectrum of the unreddened star is obscured in the spectrum of the reddened star by a fiducial mark on the raw image. Even at low resolution the spectra show a large number of lines; their identifications and spectroscopic comparisons with galactic standards will be presented elsewhere.

Table 2 Observed ($m_\lambda - m_{3100}$) colours

(Å)	SK-69-108	SK-67-108
1,300	0.604	-2.571
1,400	0.087	-2.440
1,500	-0.165	-2.381
1,600	-0.296	-2.389
1,700	-0.226	-2.126
1,800	-0.084	-2.000
1,900	0.301	-1.740
2,000	0.732	-1.523
2,100	1.079	-1.243
2,200	0.970	-1.037
2,300	0.726	-0.962
2,400	0.482	-0.934
2,500	0.308	-0.878
2,600	0.163	-0.704
2,700	0.009	-0.648
2,800	0.073	-0.412
2,900	-0.032	-0.368
3,000	-0.067	-0.249
3,100	0.000	0.000

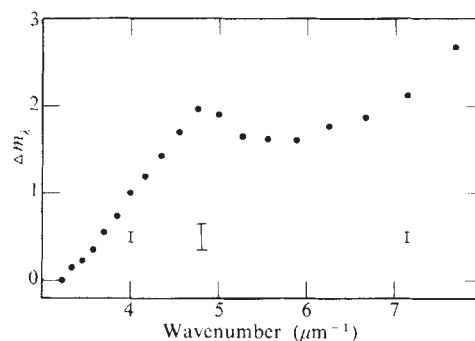


Fig. 2 The extinction law normalised to $\Delta m_\lambda = 0$ at $\lambda^{-1} = 3.23 \mu\text{m}^{-1}$ and $\Delta m_\lambda = 1$ at $\lambda^{-1} = 4.0 \mu\text{m}^{-1}$.

To improve the photometric accuracy, photometric bands ($\Delta\lambda = 100$ Å) have been constructed from 3,100 to 1,300 Å at intervals of 100 Å. The colours ($m_\lambda - m_{3100}$) for the reddened and comparison stars are given in Table 2: the errors of these colours range from 0.02 mag for the comparison star to 0.1 mag for the reddened star near 2,200 Å, where the fluxes are very low. The extinction curve Δm_λ against $\lambda^{-1} (\mu\text{m})^{-1}$ normalised to $\Delta m_\lambda = 0$ at 3,100 Å and $\Delta m_\lambda = 1$ at 2,500 Å is shown in Fig. 2. These results are preliminary so no correction has been applied for the difference in the spectral types of the two stars.

The determination of the accurate shape of the UV extinction curve in the LMC and the comparison with the galactic extinction law require a larger sample of stars of accurately known spectral types. Although this is beyond the scope of the present work, the observations presented here establish the presence of the 2,200 Å feature in the UV extinction curve in the LMC. The feature which extends from 2,500 to 1,800 Å in the spectrum of SK-69-108 is similar to what is observed in our Galaxy.

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The central source in the supernova remnant G127.1+0.5

THE discovery¹ of a very small diameter radio source with a very flat spectrum at the centre of the galactic supernova remnant G127.1+0.5 has stimulated much interest²⁻⁴. Because the central source has unusual properties, and because the *a priori* probability of finding such an object at the very centre of a supernova remnant is exceedingly low, Caswell¹ suggested that the central radio source might be emanating from the collapsed stellar remnant of the supernova that produced the surrounding shell of radio emission. This intriguing idea was pursued by Salter, Pauls and Halsam³ who showed that the extended supernova remnant is perfectly normal in shape and in radio spectrum, while the central source has a spectrum that is flat from 3 to 15 GHz at a flux density of 0.5 Jansky. A long-baseline interferometric measurement by Shaffer *et al.*⁴ at 10.65 GHz showed that the angular size of the central source is about 0.0005 arc s. We now present evidence from direct optical observation suggesting that the link between the central source and the supernova remnant is purely circumstantial.

Caswell suggested, based on positional coincidence to 0.5 arc s, that a red object of about 19 mag was identical with the source of radio emission. Inspection of the Palomar Sky Survey shows that the object is diffuse on the red plate, and invisible on the blue plate. The entire region near G127.1+0.5 shows signs of heavy obscuration.

We have made spectrophotometric measurements of the red diffuse object with the image dissector scanner at the 4-m Mayall telescope of Kitt Peak National Observatory. Our observations were obtained on 28–29 August 1978, a night of excellent seeing and transparency. On the slit-viewing television, the object was clearly nonstellar. We integrated for 4,500 s and obtained simultaneous object and sky spectra through two apertures of diameter 3.2 arc s separated by 52 arc s. The net spectrum due to the object covered the wavelength interval 4,000–7,500 Å with a resolution of about 20 Å. Figure 1 shows the distribution of flux density f_λ in $\text{erg cm}^{-2} \text{s}^{-1} \text{Å}^{-1}$ obtained by comparing the counts observed on G127.1+0.5 with those obtained on standard stars of known flux distribution.

As Fig. 1 shows, the spectrum of the object consists of a very red continuum with two broad emission blends. The two blends

are at wavelengths of 6,691 Å and 6,841 Å, and have integrated fluxes in the ratio 2.3:1. We identify the stronger, broader feature at 6,691 Å with the familiar blend of [N II] 6,548.1 Å, H α 6,562.8 Å, and [N II] 6,583.6 Å. The feature observed at 6,841 Å, we identify with the [S II] doublet 6,717.0, 6,731 Å, and we propose that the object is a galaxy.

Although the individual lines are not resolved by our instrument, we can make a reasonable estimate for the relative line strengths within the blends by using the spectrophotometry of typical radio galaxies compiled by Costero and Osterbrock⁵. Based on their work, we expect that [N II] 6,584 Å will have about the same strength as H α , and that [N II] 6,548 Å has one-third that strength. We expect that the [S II] 6,717 Å line will have the same strength as the 6,731 Å line. On that basis we compute effective wavelengths for the blends that yield a redshift $cz = 5,375 \pm 200 \text{ km s}^{-1}$. We note that the observed ratio of [N II]+H α to [S II] is about the same in this object as in the radio galaxies studied by Costero and Osterbrock.

If the object is at a distance of $\sim 100 \text{ Mpc}$, (for $H_0 = 55 \text{ km s}^{-1} \text{Mpc}^{-1}$) then it has an absolute magnitude M_v of about $-16 - A_v$. We can estimate the absorption A_v by noting that the continuum fluxes at 7,000 Å and 5,800 Å are in the ratio 2.7:1. Absolute spectrophotometry by Oke and Sandage⁶ indicates that the intrinsic ratio is about 1:1. If we attribute the reddening to dust that follows the Whitford relation^{7,8} the required extinction at the visible is $A_v = 5 \text{ mag}$. Then the absolute magnitude of the object is near $M_v = -21$, the absolute magnitude of a luminous galaxy. The presence of large extinction is corroborated by the low galactic latitude of the source ($b = +0.5^\circ$), the appearance of the region on the Palomar Sky Survey, the absence of the object from the blue plate, and the absence of short wavelength emission lines, such as [O III] 5,007 Å, that are prominent in radio galaxies.

At a distance of 100 Mpc, the radio power of the galaxy integrated to 15 GHz is about $8 \times 10^{40} \text{ erg s}^{-1}$, coming from a region of diameter 0.2 pc. For comparison, the compact source in M 87 has a diameter of 0.04 pc, and about $\frac{1}{8}$ the emitted energy per unit frequency at 15 GHz (ref. 9).

We believe that a completely consistent picture of the central source in G127.1+0.5 is that it is a luminous galaxy at a distance of $\sim 100 \text{ Mpc}$ that contains a compact and energetic radio source. Although the circumstantial evidence linking the central source to the supernova remnant is intriguing, we believe that the positional coincidence is mere chance.

Because G127.1+0.5 was the most compelling case for a compact radio source associated with a supernova remnant, the demonstration that this object is a chance event makes it seem likely that other suggested radio objects near supernova remnants may well be just the superposition of background sources on galactic remnants.

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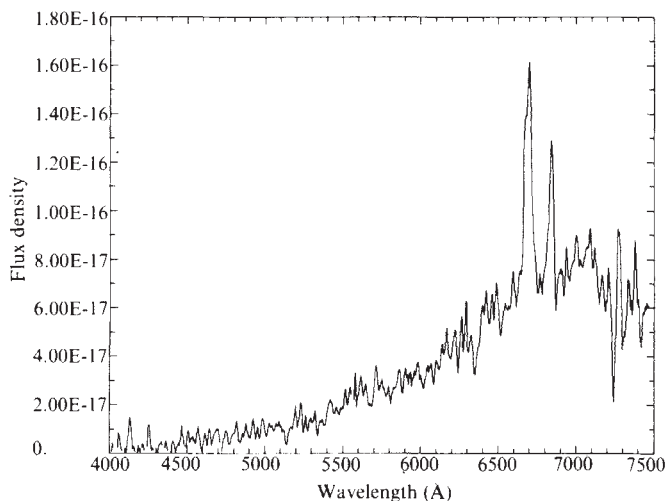
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Fig. 1 Flux density F_λ ($\text{erg cm}^{-2} \text{s}^{-1} \text{Å}^{-1}$) plotted against wavelength for the diffuse red object at the centre of the supernova remnant G127.1+0.5 ($\alpha = 01 \text{ h } 25 \text{ min } 08.3 \text{ s}$, $\delta = +62^\circ 50' 59''$ (1950)). The two broad emission features at 6,691 Å and at 6,841 Å are identified with [N II]+H α and with [S II] at a redshift of $5,375 \pm 200 \text{ km s}^{-1}$.



Magnetic field in the primitive solar nebula

METEORITES which fall to Earth's surface generally carry permanent magnetisation which probably was acquired early in their history¹. In particular, carbonaceous chondrites, which are generally assumed to be virtually unaltered relicts from the earliest Solar System processes of condensation and accretion², have apparently been magnetised in magnetic fields with intensities in the range 0.1–10 G (refs 1, 3, 4). The origin of the magnetising field has remained obscure; explanations proposed to account for the field, include a strong central solar field in the early Sun, an intense solar wind magnetic field, magnetic fields generated in large parent objects—ancestral to the meteorites—and the interstellar magnetic field transiently compressed during the Solar System's formation. So far as we know, none of these mechanisms has been shown quantitatively capable of accounting for measured meteoritic remanence⁵. We suggest here that the magnetic field recorded in the remanence of carbonaceous chondrites may have been produced by a self-excited hydro-magnetic dynamo in the gaseous preplanetary nebula from which the Solar System is thought to have formed.

The Solar System is generally thought to have formed from the collapse of gaseous interstellar matter which, because of the system's angular momentum, settled into a disk shaped nebula. Several models to describe the evolution of such a nebula have been computed^{6–10}. While these models are not unique, they do give some indication of the processes and conditions involved in the early evolution of the Solar System. For specificity, we will use the models recently computed by Cameron¹⁰, which consist of turbulent and differentially rotating, gaseous disks having characteristic radial scales of several astronomical units and characteristics half thicknesses of a few times 10^{12} cm. In the models the gas density characterising the region at several astronomical units from the centre is $\sim 3 \times 10^{-10}$ g cm⁻³ and the characteristic turbulent fluid velocity is $\sim 4 \times 10^4$ cm s⁻¹.

Turbulent motions in such a differentially rotating gas nebula can generate a large-scale magnetic field through hydro-magnetic dynamo action if the electrical conductivity of the gas is sufficiently high. At the low temperatures and high gas densities typical of a preplanetary nebula, the electron density in thermodynamic equilibrium is too low to support hydro-magnetic processes. These processes then require nonequilibrium effects to raise the electron density and electrical conductivity. The recent discovery by Lee *et al.*¹¹ of anomalously large concentrations of ²⁶Mg in some meteorite samples suggests that the short-lived radioactive isotope ²⁶Al may have been present in abundant quantities in the nebular material—the ²⁶Al produced in a nearby supernova thought to have precipitated the Solar System's formation. Cameron and Truran¹² have estimated that ~2% of the Solar System's heavy elements may have been inserted into the nebula by such an event. Ionising radiation from the decay of short-lived isotopes drives up the electrical conductivity of the nebular gas. In conditions which characterise nebular models the conductivity may be so large that the magnetic Reynolds number exceeds unity, thus coupling the behaviour of magnetic fields to the fluid motion.

In a turbulent, differentially rotating gas the efficacy of the fluid motions for generating a magnetic field can be expressed through a dimensionless dynamo number^{13,14}

$$N = \gamma \Gamma \delta^3 / \eta^2 \quad (1)$$

where γ measures the shear produced by differential rotation, Γ measures the helicity of the turbulent convection, δ is the physical scale of the magnetic field, and η is the magnetic diffusivity ($\eta = c^2/4\pi\sigma$; c is the speed of light and σ is the electrical conductivity).

Parker has investigated the regenerative modes of a hydro-magnetic dynamo operating in a differentially rotating turbulent disk of gas¹⁶ and has found that magnetic fields are produced

when $N \geq 6$. It will be shown elsewhere⁵ that this condition is satisfied in the model nebula when $\sigma \geq 400$ s⁻¹. Consolmagno and Jokipii¹⁵ suggest that ionisation produced by the decay of ²⁶Al can produce an electrical conductivity of the order of 10^3 s⁻¹ in the nebula, thus satisfying the condition for magnetic field generation.

The maximum field intensity that might be realised by the dynamo production process can be estimated. We will consider two dynamical mechanisms that limit the strength of the field. First, because the Coriolis force provides the ordering influence that produces efficient magnetic field generation in the dynamo process, an estimate of the maximum realisable field intensity can be obtained by setting the Coriolis force equal to the Lorentz force produced by the magnetic stress¹⁴. Thus, in order of magnitude

$$\rho V \Omega \sim \langle B^2 \rangle / 4\pi \delta \quad (2)$$

Taking, in equation (2), the density $\rho \approx 3 \times 10^{-10}$ g cm⁻³, the characteristic turbulent velocity $V \approx 4 \times 10^4$ cm s⁻¹, $\delta \approx 5 \times 10^{12}$ cm, the disk scale height, and Ω the Keplerian angular velocity at several astronomical units from the Sun, we find for the characteristic saturation field intensity

$$\langle B^2 \rangle^{1/2} \sim 5 \text{ G} \quad (3)$$

This is the same order of magnitude as the magnetic field which would be necessary to account for meteorite magnetic remanence.

Another mechanism which limits the field strength is ambipolar diffusion, through which the ionised gas and magnetic field slips past the neutral material¹⁷. Using the results of Consolmagno and Jokipii¹⁵ and equating the ambipolar diffusion time to the turnover time of the turbulent fluid motions one finds that ambipolar diffusion limits the further growth of the magnetic field when the field intensity rises to the order of 1 G.

The values derived here are to a large extent model-dependent and will evolve along with our ideas about the characteristics of a preplanetary nebula. However, these crude and preliminary calculations, taken with the measured remanence of primitive meteorites, suggest that a large-scale magnetic field was generated by gas motions in the preplanetary solar nebula. The intensity of this field may have been as high as 1–10 G.

Such an intense field could have had important dynamical influences on the evolution of the gaseous nebula. It is easy to see that the timescale for redistribution of angular momentum (and thus mass) driven by the magnetic stress, arising from field intensities in the range 1–10 G, is in the range 10^2 – 10^4 yr. This is the same range estimated for the evolutionary time scale of the primitive solar nebula¹⁰.

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The solar wind influences plasmasphere electron content

MAGNETOSPHERIC plasma convection has been reviewed in detail by Axford¹. A viscous-like interaction between plasma and magnetic field and magnetic-field-line merging have been suggested as mechanisms producing magnetospheric convection. In either model, convection is sustained by a dawn-dusk electric field (E_{conv}) which has been shown²⁻⁴ to depend on solar-wind speed V_{sw} . Vasyliunas² suggested a linear dependence, but Mendillo and Papagiannis³ estimated that the convection electric field varies nearly quadratically with solar wind speed—this conclusion was supported by Kivelson⁴. The convection field E_{conv} and hence solar wind speed have important roles in determining the location of plasmapause^{5,6}. Model calculations assuming enhancement of the solar wind showed the plasmapause everywhere to be located at smaller L coordinates⁷ (L is the McIlwain⁸ magnetic shell number). The location of the duskside bulge of the plasmapause has been shown to be inversely related to the solar wind speed³. The total plasmasphere electron content (N_p) is supposed to increase with increase in L , the rate of increase being dependent on the density distribution assumed and the L values under consideration⁹. Thus one expects an increase in N_p to be associated with a decrease in V_{sw} and vice versa. In this letter we give an experimental verification of this for two magnetic storms associated

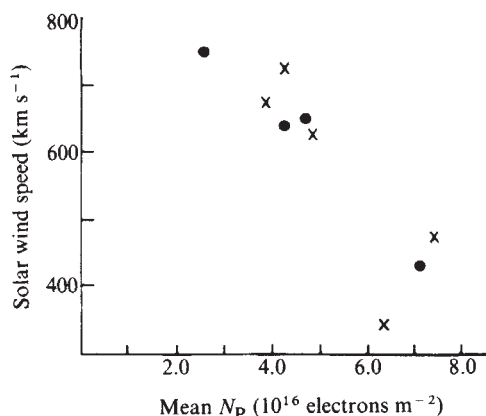


Fig. 1 Solar wind speed plotted against daily mean N_p for the two storms (see Table 1). ●, Storm of 1–5 Nov. 1975; x, storm of 28 Nov.–2 Dec. 1975.

with large changes in V_{sw} , and for which N_p and V_{sw} data are available. The results will prove useful in interpreting the disturbed time behaviour of N_p .

Electron content measurements were made between October 1975 and July 1976 at Ootacamund (11.4° N, 76.6° E, mag dip 6°) by Faraday rotation and the group delay techniques using radio beacon signals from the geostationary satellite ATS-6 at 35° E. The difference in electron contents measured by these techniques gives the electron content of the plasmasphere¹⁰. The details of the techniques and the accuracies involved are discussed by Davies *et al.*^{11,12}. The daily mean N_p values for the pre-storm and during-storm days are compared with the corresponding solar wind speeds in Table 1. For the first storm the pre-storm solar wind data are not available for the first day. Solar wind speeds are plotted against mean N_p for the corresponding days in Fig. 1. All three pre-storm days have large mean N_p values but low solar wind speeds ($\sim 400 \text{ km s}^{-1}$) whereas the during-storm solar wind speeds were greater by a factor of 1.5 with mean N_p correspondingly decreased. This is the first experimental evidence from an equatorial station confirming an inverse relationship between plasmasphere elec-

Table 1 Comparison of pre-storm and during-storm plasmasphere electron content and solar wind speed

Storms	MPO	Maximum D_{ST} (γ)	Days	V_{sw} (km s^{-1})	Daily mean $N_p \times 10^{16}$ electrons m^{-2}
1–5 Nov. 1975	2 Nov. 1100 UT	–76	1* Nov.	—	4.5
			2*	430	7.0
			3	650	4.6
			4	750	2.5
			5	640	4.2
28 Nov.–2 Dec. 1975	29 Nov. 0700 UT	–53	28* Nov.	350	6.7
			29*	475	7.3
			30	625	4.7
			1 Dec.	675	3.8
			2	725	4.2

MPO is the main phase onset; D_{ST} is the ring current intensity index.

* Pre-storm days.

tron content N_p and solar wind speed V_{sw} . The correlation coefficient between N_p and the V_{sw} is -0.92 ± 0.05 . A linear relationship between N_p and V_{sw} is obtained by the least square method and is given by $N_p = 12.5(1 - V_{\text{sw}}/1000)$ where V_{sw} is the solar wind speed in km s^{-1} ($V_{\text{sw}} \leq 750$).

Storm-time behaviour of N_p has been discussed by various workers^{13–15}. Consideration of solar wind speeds is important in interpreting the observed results and a detailed study of storm-time behaviour of N_p at Ootacamund is underway.

The experiment at Ootacamund was a joint undertaking of Physical Research Laboratory, Ahmedabad, India and NOAA, Boulder, Colorado. We thank Professors K. Davies and G. Swarup for collaboration and facilities respectively and Miss Chhaya R. Shah for computational help. The research at PRL is supported by the Department of space, Government of India.

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A new amorphous silicon-based alloy for electronic applications

THERE is a need for alternative energy sources. Photovoltaics are an attractive possibility, but their application has been limited by economic considerations in single-crystal materials, and for physical reasons such as grain boundaries in polycrystalline materials. Amorphous semiconductors are especially attractive in this regard because they are basically much less expensive than their crystalline counterparts and because they possess a direct band gap with a high value for the optical absorption coefficient. We report here the development of a new

Table 1 Comparison of the new alloy with the best silicone-hydrogen alloy fabricated

Type of amorphous alloy	Si-F-H	Si-H	Comments
Optical band gap E_0	1.65 eV	1.55 eV (refs. 11, 12)	Measured using photoconductive data
Minimum density of localised states $N(E)$	$10^{16} \text{ cm}^{-3} \text{ eV}^{-1}$	$10^{17} \text{ cm}^{-3} \text{ eV}^{-1}$	As measured by the field-effect experiments. The analysis ¹ neglects surface states and hence these numbers represent an upper limit to $N(E)$
Intrinsic behaviour	$\Delta E (\sim 0.8 \text{ eV})$ and $\sigma_{RT} (10^{-10} - 10^{-8} (\Omega \text{ cm})^{-1})$ are weakly dependent on deposition temperature between 400 and 700 K	$\Delta E (0.8 - 0.5 \text{ eV})$ and $\sigma_{RT} (10^{-11} - 10^{-7} (\Omega \text{ cm})^{-1})$ are strongly dependent on deposition temperature between 350 and 600 K (ref. 13)	σ_{RT} and ΔE refer to the room temperature conductivity and conductivity activation energy
Doping efficiency	Values of $\sigma_{RT} \sim 5 (\Omega \text{ cm})^{-1}$ and $\Delta E' \sim 0.05 \text{ eV}$ attained using very small concentrations of PH_3 (500 v.p.p.m.) and AsH_3 (50 v.p.p.m.)	Values of $\sigma_{RT} \sim 10^{-2} (\Omega \text{ cm})^{-1}$ and $\Delta E' \sim 0.20 \text{ eV}$ attained using 4×10^3 v.p.p.m. PH_3 in SiH_4	$\Delta E'$ refers to an estimate of the activation energy at room temperature by drawing a tangent to the σ - T curve
Mode of deposition	Radio frequency glow discharge in SiF_4/H_2	Radio frequency glow discharge in SiH_4 gas	
Photostructural changes	Absent	Major ⁶	
Photoconductivity σ_L	$(1-3)10^{-4} (\Omega \text{ cm})^{-1}$	$(1-3)10^{-4} (\Omega \text{ cm})^{-1}$ (ref. 14)	Measured under AM-1 incident radiation of power 90 mW cm^{-2}
Infrared peaks			Thick deposits ($\sim 5 \mu\text{m}$) made on crystalline Si substrates
2,100 cm^{-1}	Very small	Present	Usually attributed to SiH_2 (ref. 15)
2,000 cm^{-1}	Absent	Present	Usually attributed to Si-H stretch ¹⁵
1,000 cm^{-1}	Present	Present	Usually attributed to Si-O stretch ¹⁵
900 cm^{-1}	Absent	Present	Usually attributed to H-Si-H scissors ¹⁵
830 cm^{-1}	Present	Absent	Attributed to Si-F stretch ¹¹
640 cm^{-1}	Present	Present	Attributed to Si-H wag ¹⁵
Hydrogen content	$\sim 0.5\%$	$> 5\%$	Estimated from the integrated absorption coefficient by using infrared techniques ¹⁶
Fluorine content	~ 2 to 6% dependent on ratio of SiF_4/H_2	None	Estimated from ESCA measurements

alloy that eliminates the physical problems associated with the silicon-hydrogen alloys.

Amorphous semiconductors ordinarily have a large density of localised states which act as traps which lead to low values for the drift mobility and the recombination time of free carriers. For example, elemental amorphous silicon is of little technological importance because it contains a density of localised states in excess of $10^{20} \text{ cm}^{-3} \text{ eV}^{-1}$. Madan *et al.*^{1,2} reported that amorphous-silicon films decomposed from silane gas by an r.f. glow discharge and deposited on a heated substrate have a much reduced density of states at the Fermi level, of the order of $10^{17} \text{ cm}^{-3} \text{ eV}^{-1}$. This material has subsequently been shown to be an amorphous silicon-hydrogen alloy³. In addition, Spear and Le Comber⁴ showed that this alloy could be doped with phosphorus and boron; Carlson and Wronski⁵ further demonstrated that it had a sufficiently high carrier lifetime to yield solar cells of up to 5.5% efficiency. Nevertheless, the still relatively high density of localised states throughout the gap leads to inefficient doping and limits the carrier lifetime. In addition, undesirable photostructural changes have been observed⁶ and the material effuses hydrogen above 450 °C (ref. 3). Despite many efforts, there has been no success in overcoming these basic problems.

The new alloy has silicon and fluorine as its main structural components. This alloy is multi-elemental and includes hydrogen, and can also include other elements such as oxygen without deleterious effects. The steric chemical and electrical properties associated with our new alloys will be described elsewhere^{7,8}. Table 1, which condenses data taken from more than 600 sample depositions, compares our Si-F-H alloy (fabricated from the gas ratio of $\text{SiF}_4/\text{H}_2 = 10/1$) with the best Si-H alloy.

Table 1 shows that our material is in almost all properties, except for photoconductivity, distinctly different from the best Si-H alloy which makes it attractive for electronic device applications. Of special interest is the fact that the intrinsic properties of our material are only weakly dependent on the substrate

temperature, much like multi-component chalcogenide glasses, and in sharp distinction from the strong dependence observed in silane-decomposed films.

With regard to the infrared spectrum, it is important that the peak at $2,000 \text{ cm}^{-1}$ corresponding to the silicon-hydrogen stretch does not appear in our films. The peak at $2,100 \text{ cm}^{-1}$ is ordinarily assigned to the SiH_2 group which has a wag at 640 cm^{-1} and a H-Si-H scissors mode at 900 cm^{-1} . The scissors mode is also absent in our film. Hydrogen through its various bonding configurations with other elements can be responsible for localised states in the gap^{9,10}. A full interpretation of this point and the above data will be given elsewhere¹¹.

The new alloy reported here is superior to silane-based films for electronic applications. In particular, there is a much lower density of states at the Fermi level. This leads to much larger doping efficiencies. In fact, effective n-type doping has been achieved with only 50 v.p.p.m. AsH_3 in the premix. Furthermore, no photostructural effects have been observed. Because of its highly desirable characteristics, this material seems to have a potential for general semiconducting applications as well as for photovoltaics.

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A new structural model for transition metal-metalloid glasses

CONCEPTUAL notions of the structure of metallic alloy glasses have generally developed from purely geometrical sphere-packing models proposed for liquid¹ and glassy^{2,3} monoatomic metals. The assumption on which these models are based is that the glass consists of a random arrangement of spherical atoms of each element—the so-called dense-random-packed (DRP) model. Glassy 'metallic' alloys of two or more chemically dissimilar elements can also be represented in this way, local atomic arrangements arising by chance, constrained only by geometrical factors such as the ratios of atomic radii. Construction of the model described here is based on an alternative principle commonly observed in amorphous solids of other types, namely that local structure is well-defined and almost always identical to that found in crystalline forms of the material. The properties of such a 'stereochemically designed' model have been computed and are found to be in reasonable agreement with high resolution neutron scattering data for glassy palladium-silicon alloys.

Metallic alloy glasses can be subdivided into two broad categories: alloys of two or more metallic elements (metal-metal glasses) and those systems in which a non-metallic or semi-metallic (metalloid) element is essential for glass formation. Within the latter category, the binary and ternary alloys of the transition metals, particularly Fe, Co, Ni, Pd and the non-metallic elements B, C, P and Si comprise the most important series, commercially and scientifically. Dense-random-packed structures for these glasses allow the non-metallic atom to occupy polyhedral cavities of several different types, only one of which resembles the local coordination observed in the crystalline state. Structures represented by this model are essentially different from those of most other types of glass: in oxides and chalcogenides, amorphous elemental and compound semiconductors, organic 'molecular' and polymeric glasses, the nearest-neighbour coordination and local symmetry, at least, are well defined. It is clearly incorrect, for example, to represent a glassy silicate as anything other than a non-periodic arrangement of relatively undistorted SiO₄ tetrahedra^{4,5}, the latter being, of course, the local structural units on which nearly all simple silicate lattices are based.

Fig. 1 *a*, Regular trigonal prismatic coordination; and *b*, edge-sharing of polyhedra observed in the Fe₃C₂ cementite structure. Note that the M_I atom G for the upper prism occupies an M_{II} site in relation to the lower.

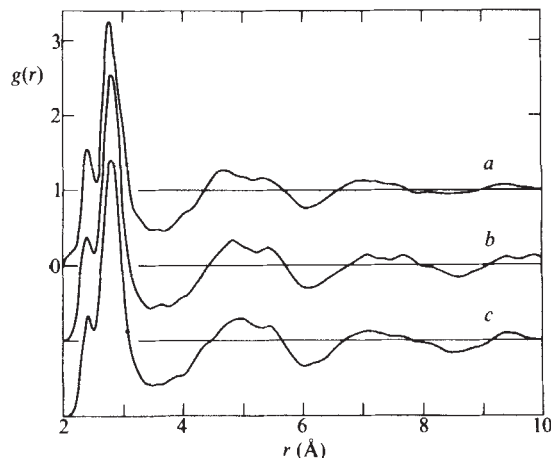
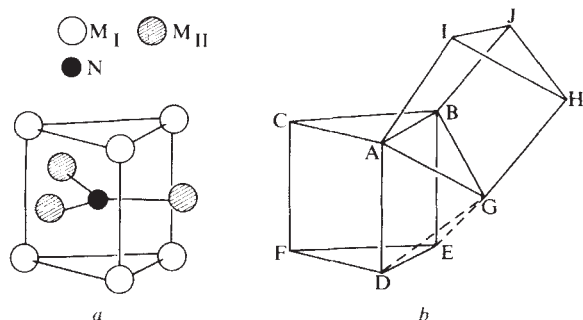


Fig. 2 Pair correlation function $\rho(r)/\rho_0$ for glassy Pd₄Si, where $\rho(r)$ is the atomic density and ρ_0 the average density. *a*, Experimental data derived from neutron scattering measurements¹² with $Q_{\max} = 25 \text{ \AA}^{-1}$ ($Q = 4\pi \sin \theta/\lambda$). Curves *b* and *c* refer to spherical cores of the model of radius 8.6 and 10 Å respectively. The latter cluster is not fully dense but nonetheless gives a better representation of $g(r)$ beyond $\sim 5 \text{ \AA}$ where the effects of finite model size become important. Computed curves have been convoluted with gaussian broadening functions to simulate the effects of thermal vibration and transformation of experimental scattering data over a limited range of Q . Values were chosen to reproduce the breadth of each component of the split first peak at 2.4–2.9 Å (half widths are 0.094 Å for Si-Pd and Si-Si, and 0.14 Å for Pd-Pd correlations).

Local structure in the borides, carbides, phosphides and silicides of most of the transition metals which form glasses on quenching from the melt is also well defined in the crystalline state⁶. The coordination polyhedron of the non-metallic element, N, consists of a subshell of six metal atoms, M_I, forming a somewhat distorted trigonal prism with three further M_{II} atoms capping the square faces at somewhat larger and even more variable M–N distances (Fig. 1*a*). Do the glassy forms of these materials therefore also consist of non-periodic arrangements of such units? Are glasses, formed from palladium and silicon or iron and boron, random amorphous alloys of the two pairs of elements, or glassy silicides and borides with well defined local symmetry similar to that found in the crystalline forms?

The influence of localised, possibly directional chemical bonding, the formation of relatively large groups of atoms with low symmetry, are relevant factors—if only in a qualitative sense—to theories of glass formation and stability. Arrangements of spherical atoms into a periodic array involves relatively simple processes, whereas ordered packing of large asymmetric 'molecular' fragments requires a series of correlated motions. Glass formation is therefore more likely in the latter case, as has been noted elsewhere⁷. Alternative theories stress the stability gained when atoms of one element fit within cavities formed by random packing of the other⁸ or collective properties of the valence electrons—specifically, the reciprocal space relationship between the Fermi momentum and the first peak of the structure factor⁹. In neither of the latter treatments is bonding considered explicitly. The importance of each of these three factors in particular alloy systems is a source of controversy¹⁰.

This paper describes properties of an atomic model for metal-metalloid glasses in which trigonal prismatic NM₆ units are connected in an essentially random manner, with an edge-sharing arrangement (Fig. 1*b*) observed in many of the crystalline forms of these materials, notably the Fe₃C₂ cementite structure. A physical model, built from 3-cm polystyrene spheres, was used to guide a computer program which generated the atomic coordinates of a cluster with a radius of about 12 Å containing 434 M and 108 N atoms. Atomic coordinates are unrealistic at this stage for two reasons. First, regular, undistorted trigonal prisms were used to simplify the construction algorithm; however, N atoms are generally too large to fit within

a regular trigonal prism which must therefore distort. Second, the physical model allows no positional adjustment once units are fixed in place and it is impossible therefore to build a model which does not contain relatively large voids on the one hand and 'compressed' atoms on the other, although excessive overlap of atoms can be avoided in both the hand-built and computer-generated models. Coordinates were refined by moving atoms sequentially to minimise the energy calculated using a Lennard-Jones pair potential

$$V_i = 4\epsilon_{ij} \sum_j (R_{ij}^{12} - R_{ij}^6)$$

Here, V_i is the energy of atom i , $R_{ij} = 2^{-1/6} R_{ij}^0 / r_{ij}$, where r_{ij} is the actual distance between the atoms i and j and R_{ij}^0 the equilibrium distance. The quantities ϵ_{ij} are approximately proportional to weighted sums of the cohesive energies of each element and

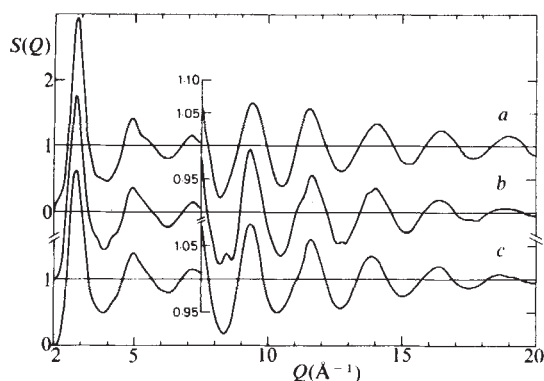


Fig. 3 Neutron structure factors $S(Q)$ for a -Pd₄Si: labelling as for Fig. 2. The computed curves were obtained by a sine transform of real-space data (over the range 0–15 Å) modified by a weighting function $\sin(\pi r/12)/(\pi r/12)$ introduced to suppress errors in the radial distribution function beyond about 8 Å.

have been obtained from the tabulation of Gschneider¹¹. In the summation, j includes all nearest-neighbour atoms: for M–M pairs, atoms within a sphere of radius $3.2 R_M^0$ centred on the origin atom are included where R_M^0 is the radius of the metal atom. Atoms are free to move in and out of this sphere as energy minimisation proceeds. For N–M pairs, however, trigonal prismatic local coordination is emphasised by attempting to keep the local environment of the N atoms invariant, the program attempts to retain the original six nearest-neighbour M neighbours of each N atom throughout the calculation. Moreover, the average bond length is set to the N–M_I distance found in crystals. Further M_{II} metal neighbours lying with $2.4 R_M^0$ are included in the summation: these atoms are unconstrained and a larger equilibrium N–M_{II} distance is assumed.

Figure 2 shows the pair correlation function $g(r) = \rho(r)/\rho_0$ computed for glassy Pd₄Si, compared with experimental data derived from neutron scattering measurements⁹. Here, $\rho(r)$ is the atomic radial density function and ρ_0 the average atomic density. The function $g(r)$ has been calculated for spherical cores of radius 8.6 and 10 Å. As the model is small, the average density is a function of the cluster radius—a core of radius 8.6 Å is almost fully dense whereas at 10 Å, parts of the spherical core lie beyond the edge of the model. The average density, $10.4 \pm 0.2 \text{ g cm}^{-3}$ is in good agreement with experimental values for a -Pd₄Si of 10.25 (ref. 3) and 10.5 g cm^{-3} (Suzuki, personal communication). The neutron scattering factor, $S(Q) = 1 + 1/Q \int_0^\infty 4\pi r(\rho(r) - \rho_0) \sin Qr \, dr$ is shown in Fig. 3.

Although there are some discrepancies between computed and experimental $g(r)$ values—notably the height of the first (Si–Pd) peak at 2.4 Å and the position of the first component of the second split peak at 4.8 Å, overall agreement is reasonable. Agreement between experimental and computed neutron scattering data, Fig. 3, is excellent, the somewhat more rapid decay of oscillations in $S(Q)$ for the model beyond about 17 Å^{-1} indicating greater order in the real glass.

This work cannot conclusively resolve the issue of defined (chemical) compared with random (geometrical) local structure, scattering data are almost certainly insufficiently sensitive to discriminate between the two models. However, the defined local coordination model apparently gives a good representation of transition metal–metalloid glasses, especially those which have been quenched relatively slowly or have been subsequently annealed and thus approximate to the minimum free energy amorphous state—an 'idealised' glassy state—the vitreous equivalent of the perfect single crystal. In this context it is interesting that atomic coordinates can be generated for a model in which the local coordination around silicon atoms is less well defined (by removing the distinction between N–M_I and N–M_{II} distances mentioned above). Changes in $S(Q)$ thus introduced give a good representation of experimental changes in X-ray scattering observed when metallic glasses are annealed at temperatures below their glass transition or crystallisation temperatures^{13,14}, the more ordered model corresponding most closely to an annealed glass.

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Transient effects in laboratory measurements of compressional wave velocity and electrical conductivity

LEE *et al.*¹ demonstrated the effect of temperature on the electrical conductivity of high grade saturated metamorphic rocks, when held at constant effective pressures. Apart from initially showing an increase in conductivity with temperature, it was demonstrated that conductivity at higher temperatures was time dependent. When a sample was held at a constant temperature ($\approx 250^\circ\text{C}$) the conductivity decreased exponentially with time, so that after ~ 10 h, there was little further change observed. As the temperature was lowered the conductivity decreased from this steady state value, and with further thermal recycling the conductivity was recycled along a path

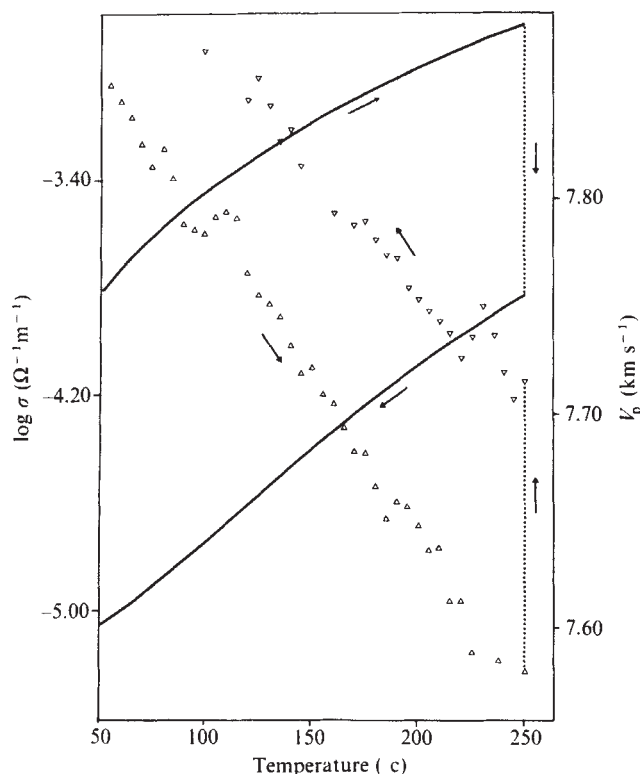


Fig. 1 Electrical conductivity and compressional wave velocities for a saturated (0.5 M NaCl) sample of ultramafic rock as a function of temperature. The electrical conductivity is described by the solid line and compressional velocity by triangles, inverted for decreasing temperature. The transient effect at elevated temperatures is signified by dotted lines. Confining pressure, 0.35 GPa; pore pressure, 0.10 GPa.

quite different from that taken with the initial temperature increase. The results implied that due regard should be paid to this transient effect when making laboratory electrical measurements on rock samples in simulated upper crustal conditions. Time-dependent effects at elevated temperatures have also been observed in laboratory measurements of compressional wave velocities^{2,3}. Spencer and Nur² in their study of the Westerly Granite showed that thermal recycling at elevated pressures tended to produce an irreversible increase in compressional velocities. Further investigation demonstrated a systematic time-dependent increase of velocity while the sample

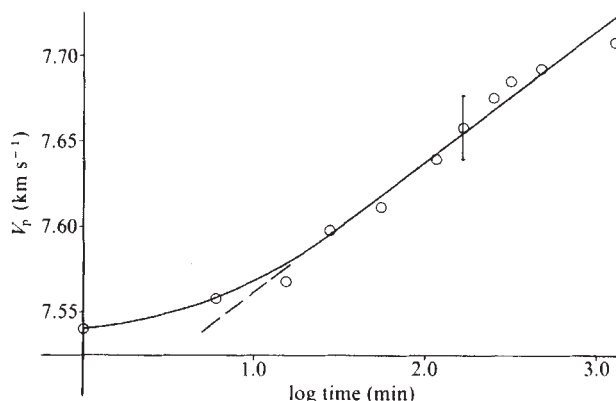


Fig. 2 Compressional velocity as a function of log time for a saturated (0.5 M NaCl) sample of ultramafic rock while held at 250 °C. Confining pressure, 0.35 GPa; pore pressure, 0.10 GPa.

was held at a fixed elevated temperature (495 °C). We report here the direct comparison of these transient variations of electrical conductivity and compressional velocity. In our experiments we have measured these parameters simultaneously on the same sample to ensure that any variables such as pore pressure, confining pressure and temperature are identical. Any comparison of separate measurements of conductivity and velocity on the same sample in different experiments is unsatisfactory due to the irreversible nature of the process being studied. Even if two samples from the same rock are used there may be significant differences in cracks and mineralogy, which would give erroneous results.

The apparatus we used is similar to that of Lee *et al.*¹, except for the introduction of lithium niobate transducers within the metal electrodes. A pulse transmission method was used², with a 60 V (0.5 mHz) square wave applied to the input transducer and the resultant output signal (received by the output transducer) displayed on a dual-beam oscilloscope. The transit time through the sample was measured by a Hewlett-Packard timer counter triggered by the input pulse and terminated by a marker pulse, visually aligned with the first arrival of the output signal. This method introduced an error of ~0.5% between different measurements and an absolute error of ~3% in velocity readings.

A wide range of rocks including granite, quartzite, gneiss, schist, gabbro and basalt have been studied at elevated pressures and temperatures (confining pressure 0.35 GPa; pore pressure 0.1 GPa; temperature up to 250 °C). All have shown the transient decrease in conductivity described by Lee *et al.*¹, and with this a corresponding velocity increase.

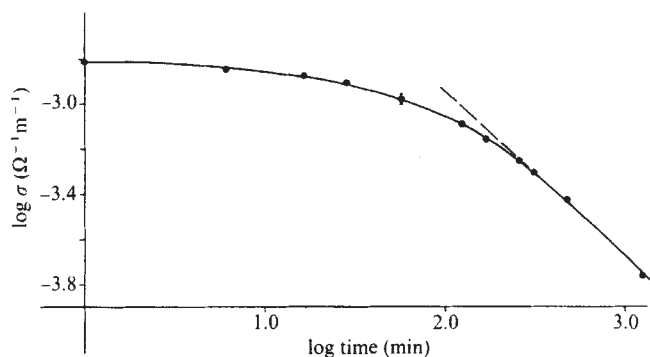


Fig. 3 Log conductivity as a function of log time for the same sample of ultramafic rock as that described in Fig. 2. The measurements of conductivity were made simultaneously with the velocity. Temperature, 250 °C; confining pressure, 0.35 GPa; pore pressure, 0.10 GPa.

A clear example of these effects are shown in Fig. 1, which depicts the first thermal cycle on a sample of ultramafic rock. While the sample was held at 250 °C there was an initial rapid increase in velocity with time ($\dot{V}_p = 4 \times 10^{-4} \text{ km s}^{-1}/\text{min}$). The rate of increase in velocity, $\dot{V}_p$, decreased inversely with time so that after ~48 h a steady state maximum value of velocity was obtained. After the initial increase a linear relationship is suggested between the compressional wave velocity and log time (in min) (Fig. 2): $V_p \propto \log(\text{time})$.

The results of the corresponding conductivity measurements are shown in Fig. 3. Lee *et al.* suggested that after the initial drop in conductivity (where uncertainty arises about the precise onset time): $\log \sigma \propto \log(\text{time})$.

One might expect therefore a close relationship between the change in compressional wave velocity and the change in electrical conductivity. However, a direct comparison of these two parameters is unsatisfactory, due largely to the magnitude of the increase in velocity at the later stage of the transient effect being small compared to the observational error. In any case, Figs 2

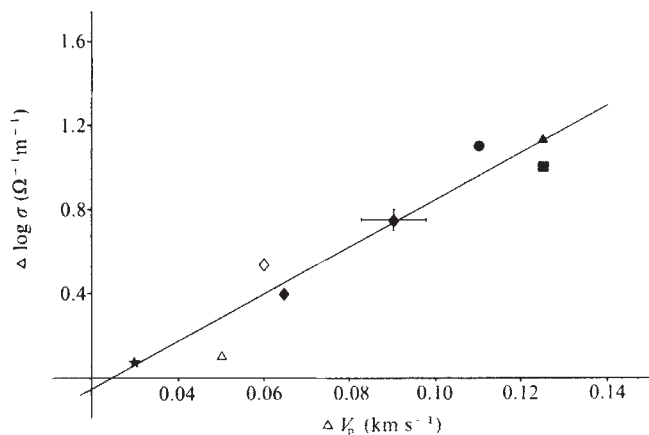


Fig. 4 Comparison of the magnitude of the transient compressional wave velocity and log conductivity effects over a period of 17 h for several rock types. Temperature, 250 °C; confining pressure, 0.30 GPa; pore pressure, 0.10 GPa; pore fluid, 0.5 M NaCl. ▲, Saturated granite; △, dry granite; ■, peridotite; ●, garnet mica schist; ◆, gabbro; ◇, gneiss; ★, quartzite.

and 3 suggest that the relationship may be more complex, in that the onset times of the linear segments are quite different for the two parameters. Comparison of the magnitude of the transient effect for several rock types and over a fixed time interval (Fig. 4) does show, however, a general relationship between the change in velocity and the change in (log) conductivity.

Spencer and Nur² suggested that the variation in velocity with time might be due to a solution of quartz in supercritical pore water. An increased content of silica in the pore fluid might increase the bulk modulus or viscosity of the fluid and hence the compressional velocity in the aggregate. However, increased silica content would also increase the electrical conductivity⁶, whereas our observations of the transient effect associate increasing velocity with decreasing conductivity.

Lee *et al.*¹ suggested that the transient behaviour of electrical conductivity might be a sensitive indicator of a creep process. A primary creep⁴ mechanism at these elevated temperatures and high effective pressures would result in the further closure of low aspect ratio microcracks, resulting in a significant drop in electrical conductivity⁶. It would also produce a marked decrease in compressibility of the sample⁵ giving an increase in compressional wave velocity.

Our results lead us to believe that elevation of temperatures above 250 °C at a high effective pressure produces an irreversible process within the samples. This process must affect a large group of physical parameters and consequently be important in any discussion of experimental work when upper crustal conditions are simulated. In particular, measurements of temperature effects on conductivity and velocities should require a thorough thermal recycling study.

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Nuclear magnetic resonance spectroscopy of humic materials

ALTHOUGH humic substances are some of the most important naturally occurring macromolecules in both soil and aqueous environments, their structures are still unknown. Our understanding of the components which comprise humic mixtures has been confined to the analysis of only minor portions (5–10%) of the total humic entity. It is, however, the whole humic aggregate, most of which is structurally unknown, which plays an active nutritional or transport role in nature. When the major fraction of humic matter, the alkali soluble substances, are studied, only poorly defined spectra have been obtained. Infrared, ¹H-NMR and ¹³C-NMR spectroscopic techniques have yielded unresolved spectra in which little detail can be distinguished^{1–5}. We report here that, using large magnetic fields and very high frequency (270 MHz) Fourier transform ¹H-NMR instrumentation, we have found that a wealth of detail can be obtained on the structure of humic solutions. A convolution difference method⁶ allows discrete signals to be detected above the general broad band absorptions in much the same way as important structural and dynamic information concerning proteins has been gathered.

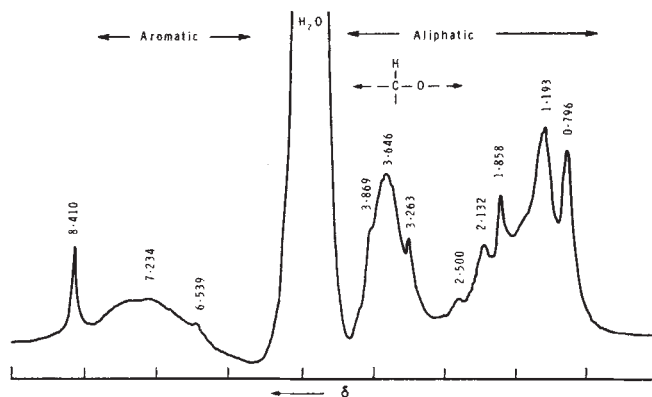


Fig. 1 270 MHz ¹H-NMR spectrum of Wakanui humic material in NaOH; chemical shifts are in p.p.m from internal TMS. They were measured with respect to external TMS and corrected. A pulse of 10 μs (85° tilt angle) was used to view a spectral window of 3.6 kHz in 8 K data points. Humic material was prepared by extraction of soil with 0.1 mol dm⁻³ NaOH. The mixture was acidified and the precipitate redissolved in NaOH for spectral examination.

The 270 MHz Fourier transform proton spectra of alkali soluble Wakanui silt loam (Wakanui silt loam—parent material—late Pleistocene loess imperfectly drained with seasonal high water table. pH = 5.8; C% 4.0, N% 0.35; C/N = 11.4, C.E.C. (me%) 23.4. New Zealand classification—yellow-grey earth) 'A' horizon material is shown in Fig. 1. The peak at 8.4δ arises from formate ion. This is confirmed by the signal at 167δ in the ¹³C-NMR spectrum. From the integrated spectrum it is possible to estimate the fraction of protons, excluding exchangeable protons, which are aromatic (H_{ar}^{*}), and the fraction which is aliphatic (H_{al}^{*}).

The Brown and Ladner equation⁷, $f_a = [(C/H) - 0.5H_{al}^*]/(C/H)^{-1}$, (where C/H is the carbon to hydrogen mol ratio), has been successfully applied to a range of carbonaceous mixtures for measuring the fraction of aromatic to total carbon (f_a) in the mixture. Recently, its basic assumption that the mol ratio of aliphatic hydrogen to aliphatic carbon is of the order of two, has been confirmed by ¹³C-NMR spectroscopy⁸. ¹³C-NMR spectroscopy of humic substances is still in its

infancy⁵ and when applied the Brown and Ladner assumptions may not be valid because of the predominance of COOH groups in humic substances. However, if f_a is redefined as f'_a , the fraction of aromatic carbon plus carboxylic carbon to total carbon, it becomes a useful parameter for describing the structure of humic substances.

Our results indicate that 65% of the carbon in Wakanui silt loam extract is aromatic and carboxylic acid carbon. The parameters f'_a and H_{ar}^* are 0.65 and 0.16 respectively. For moss peat soil, H_{ar}^* is around the same value but f'_a is much lower (0.50). This contrasts with a podsol 'A' horizon of $H_{ar}^* = 0.43$, $f'_a = 0.72$. Use of Brown and Ladner's parameters, σ , the degree of substitution, and H_{aru}/C_{ar} , the atomic hydrogen to carbon ratio of the hypothetically unsubstituted aromatic material is not justified because large amounts of oxygen-bonded aliphatic material are present in our extracts.

The combination of a low H_{ar}^* and high f'_a value recorded for loam extract, indicates that the solution contains aromatic material which is polycyclic or heavily substituted monocyclic. The presence of aromatic 1H -NMR absorptions at $>8.1 \delta$ can be assigned to sterically hindered protons characteristic of many polycyclics. In our spectra they have only a very minor contribution to the total aromatic absorption.

From the combustion analysis, we were able to show that the mol ratio of hydrogen to nitrogen in this substrate is 12.4. In view of the known forms of nitrogen in humic materials¹, most of the nitrogen is probably bound to one or less protons. The contribution of nitrogen-bonded protons must then be $\leq 8\%$.

Of the rest, it seems from the absorptions around 3.6δ that a large proportion (32%) of the aliphatic, or 27% of the total (excluding exchangeable) protons, is bound to carbon α to oxygen. The predominance of such aliphatic oxygen-bound carbon suggests a major proportion of sugar-like components or other polyhydroxy or polyether materials. This is supported by

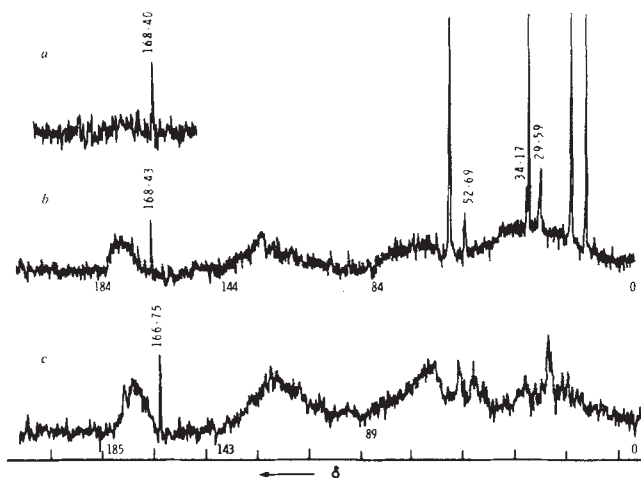


Fig. 2 ^{13}C NMR spectra of humic materials: a, Mangaroa humic acid peak at 168δ ; b, Mangaroa fulvic acid; c, Wakanui humic acid. Signals were observed relative to external TMS and corrected relative to internal TMS. Observed signals for Wakanui material were: 179.69, 184–169 (broad, halfwidth = 9, $\delta_{max} = 177.06$, COOH) 166.75 (strong), 143–106 (broad, halfwidth = 17, $\delta_{max} = 127.02$, aryl) 84–65 (broad, halfwidth = 13, $\delta_{max} = 70.27$, RZHCOS, Z = R, H) 59.71, 59.3, 54.35, 35.80, 27.58 (strong, CH_2) 26.61, 22.6, 20.4. Observed signals for Mangaroa fulvic acid were 185–171 (broad, halfwidth = 8, $\delta_{max} = 181.24$) 168.43 (strong), 144–104 (broad, halfwidth = 10, $\delta_{max} = 128.82$) 89–63 (broad, halfwidth = 16, $\delta_{max} = 74.54$) 52.69 (strong), 34.17 (strong), 29.59 (strong) 55–8 (broad). 1-butanol peaks have not been listed. Spectra were recorded at 67.89 MHz with a 22 μs pulse (60° tilt angle). Acquisition time was 1.08 s for a 15 kHz spectral window in 32 K of memory and a delay of 1–2 s. Data were accumulated over 12 h.

the ^{13}C -NMR spectrum (Fig. 2c) which also indicates COOH, aromatic and alkyl groups⁵.

Our spectra confirm the previous results^{4,9–12} that alkyl chains make important contributions to humic substances. The signal at around 0.796δ can be ascribed to $CH_3 \gamma$ or further from aromatic rings. Polymethylene is present probably because of its low biodegradability⁹.

In humic materials isolated from river sources, we observed the same general patterns although the $H-C-O-$ absorptions were not as distinct, and protonated aromatic material represented only a very minor contribution. We were able to show by attenuation that the fine structure of the aromatic absorptions from both potable (Akatawara) and swamp water (Mangaroa) sources were very similar (see legend

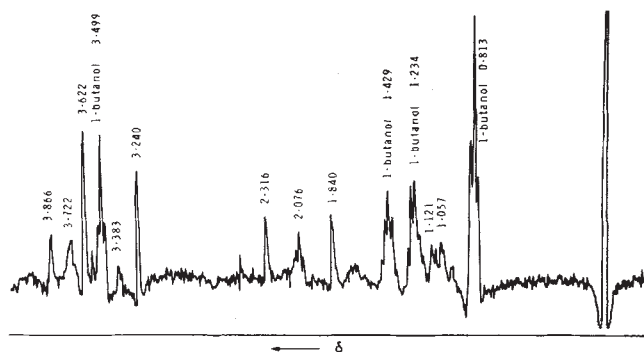


Fig. 3 Convolution difference spectrum of Mangaroa humic material. The signals due to 1-butanol arise from solvent tightly bound to the humic material which we were unable to remove on drying to constant weight. Samples were prepared by common water chemistry techniques¹⁵ involving filtration, concentration, acidification and extraction of 1-butanol-soluble material. Mangaroa humic material: source—Black Creek, Mangaroa Valley, New Zealand. pH = 6.5; conductivity, 8.0 mS m^{-1} ; absorbance, 0.990 at 270 nm; colour, 300 Hazen units. Akatawara humic material: source—Akatawara River. Upper Hutt, New Zealand (typical potable water). pH = 6.9; conductivity 7.1 mS m^{-1} ; absorbance 0.210 at 270 nm; colour 40 Hazen units.

to Fig. 3 for a description of the waters). Application of a resolution-enhancement technique⁶ which uses the difference between the broadened and resolved signals revealed detailed resonance information (Fig. 3). For example, Mangaroa humic acid isolated from a typical swamp water source in New Zealand showed at least six clear-cut aliphatic resonances which could be followed by, say, chemical modification of the humic material. Similarly, fine structure was found for samples from a potable (Akatawara) aqueous source.

Our results agree with the increasing amount of evidence^{4,5,9,13} that humic materials are not coal or lignin like in structure. The presence of large amounts of aliphatic polyhydroxy groups explains both the insolubility of humic materials in organic solvents and the behaviour of these substances to methylation^{1,14}. Of particular interest is a methylated chloroform-soluble subfraction of a fulvic acid isolated by Schnitzer¹⁰; this has similar important absorptions around 3.6δ . The subfraction has a number average molecular weight of 821 g mol^{-1} and possibly represents a small fragment of humic material of a type not unlike some of the macromolecules observed in our extracts. Some of the signals may not arise from methylation.

In contrast to other branches of environmental science, structural studies on soil and aqueous organic matter have always been hindered by a lack of available analytical tools. Our results reported briefly here show that at last this tool has arrived and that the next decade will see a great increase in studies using

NMR techniques which will throw new light on the chemistry of these elusive materials.

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Desert varnish: evidence for cyclic deposition of manganese

WE have found evidence that desert-varnish coatings on rocks in the western US consist of micron-scale alternating dark and light layers that differ in manganese and iron content. We report here the first quantitative electron-microprobe analyses of desert varnish, that also show the chemical variations in these layers. In the field and laboratory we obtained new evidence that varnish covers a fresh substrate by nucleation of micron-sized patches that grow and coalesce.

Varnish-coated samples from Arizona, Utah and Idaho were studied in the field and in the laboratory. Host rocks include

basalt, granite, quartzite, limestone, sandstone, caliche, quartz and opal. Varnish coatings range in thickness from a few micrometres to hundreds of micrometres, and characteristically are rich in manganese and iron^{1–13}. Varnishes from widely separated localities have similar chemical, mineralogical, morphological and optical properties, and are compositionally independent of the host rock.

Ultra-thin (~10 µm) sections cut normal to the rock surfaces were prepared (by us and by R. Beauchamp). Sections from the three localities reveal alternating black and light-red layers that range in thickness from 0.25 to ≥20 µm (Fig. 1). The layers are typically a few micrometres thick. Mound-like or botryoidal structures are common, and within these structures the thicknesses of the layers are highly variable. Many layers can be traced laterally for at least several millimetres, except within the botryoidal structures where layers may extend for only tens of micrometres. Sections from single boulders show correlations between major layers in the varnish. Because of the difficulty of preparing ultra-thin sections (without losing the soft varnish) we have insufficient samples to say whether varnish layers can be correlated among samples on a local or regional scale.

Representative electron-microprobe analyses of varnish layers are given in Table 1. The areas analysed are shown in Fig. 1a. An analysis of the feldspathic matrix of the basalt substrate below the varnish is included in Table 1. Although it has long been known that desert varnish is rich in manganese^{12,13} our data show that the manganese is concentrated in the optically opaque or dark layers (~20 wt% MnO₂) and that the adjacent red layers are relatively Mn-poor (~3 wt% MnO₂). The red layers are enriched in Fe₂O₃, SiO₂, Al₂O₃ and K₂O, whereas the dark layers contain more CaO. Other elements such as Ti, Mg, Na, P and S seem to be fairly evenly distributed throughout the coating.

In thin sections we can distinguish optically between the layered varnish and pockets of detrital material (Fig. 1b) consisting largely of clay with minor amounts of feldspar, quartz and hematite. The detritus typically makes up <30% of the coating. It occurs between botryoidal mounds where it seems to have been trapped by the growing varnish. In Fig. 2 the scanning electron microscope shows the botryoidal surface of the same varnish sample in Fig. 1. Figure 2 also shows random detrital minerals, and illustrates how grains can accumulate between the mound-like structures. These morphological relationships imply that analyses of scrapings of desert varnish include both layered varnish and trapped detritus.

The mineralogy of the varnish is not yet completely understood. Potter and Rossman^{2–4} using scrapings of varnish from the Mohave Desert, California, concluded on the basis of mid-

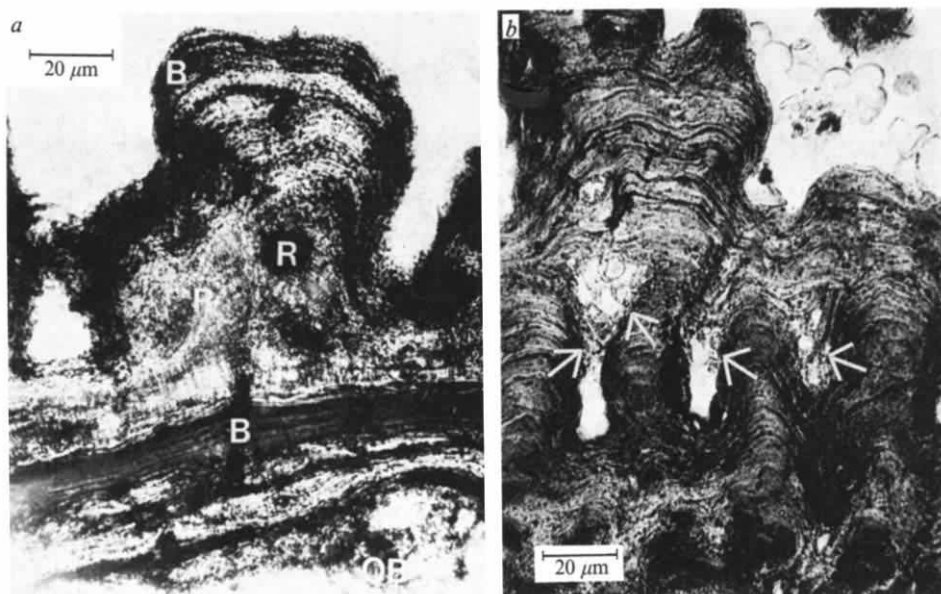


Fig. 1 *a*, Photomicrograph of Arizona desert varnish in a thin section cut normal to the surface of the sample. The varnish coats an olivine basalt (OB). Dark (black) (B) and light (red) (R) layers express mainly variations in Mn/Fe ratio (see Table 1) and imply alternating conditions of deposition. Mound-like structures are similar to those in Fig. 2. The closely spaced black bands at B are where electron microprobe analyses were made. (R) marks analyses representing an Mn-depleted and Fe-rich zone. The dark spot and lines are artefacts from the electron microprobe beam. Plane polarised light. *b*, Similar to (*a*) Arrows indicate pockets of detrital clays with minor feldspar, zeolite (?), quartz and hematite.

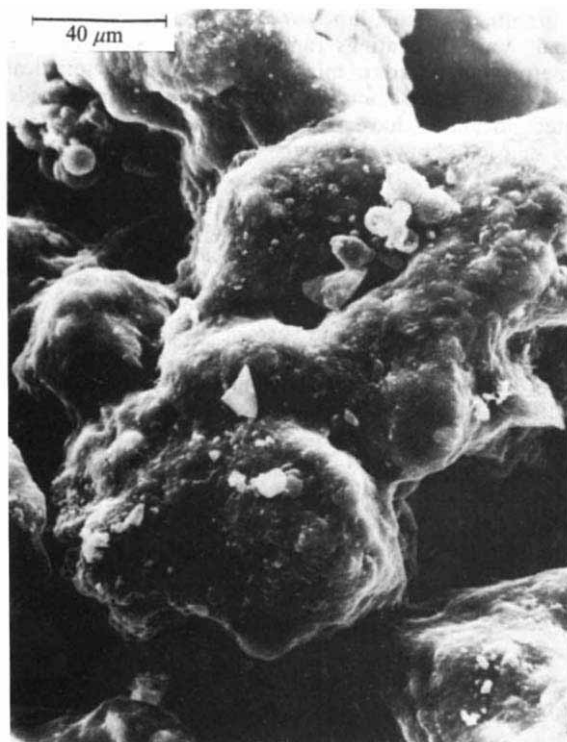


Fig. 2 Scanning electron micrograph of typical desert-varnish surface. Mound-like or botryoidal structures enlarge, coalesce, and are succeeded by new mounds as varnish accretes. Windblown-dust particles and microfloras visible on surface collect primarily in depressions, and become trapped in the varnish layer. Depressions may also shelter microorganisms. Cross-sections through a similar varnish surface are shown in Fig. 1.

IR spectroscopic measurements of chemical extracts that the main minerals are mixed-layer illite-montmorillonite clays with an intimate mixture of birnessite $[(\text{Na}, \text{Ca}, \text{K}) \text{Mn}_7\text{O}_{14} \cdot 3\text{H}_2\text{O}]$ and hematite (Fe_2O_3). However, they found no X-ray powder-diffraction evidence for oxides. Our X-ray diffraction measurements also did not reveal definite peaks of oxides, and we agree with Potter and Rossman that any oxides present must be very fine grained ($<0.1 \mu\text{m}$). We found relatively strong peaks at ~ 12 , ~ 10 , ~ 4.50 , ~ 3.36 and $2.57 \text{ \AA}$, which may be

Table 1 *a*, Electron microprobe analyses of varnish layers from Arizona*; *b*, total water% in scraped Arizona varnish and in wind-deposited soil

<i>a</i>							
	Basalt	Band area 1		Band area 2		Band area 3	
Oxide	Substrate†	Black	Red	Black	Red	Black	Red
SiO ₂	54.21	26.93	34.11	26.74	31.10	24.25	30.83
TiO ₂	0.25	0.48	0.77	0.68	0.53	0.67	0.58
Al ₂ O ₃	27.14	18.78	24.84	21.25	26.45	20.93	26.16
Fe ₂ O ₃ ⁽²⁾	2.08	17.41	23.54	18.44	24.51	13.45	24.83
MnO ₂ ⁽²⁾	0.00	20.46	2.92	17.52	2.47	24.41	2.94
MgO	0.14	2.44	2.24	1.92	3.42	1.70	2.91
CaO	9.36	0.98	0.50	1.11	0.76	1.25	0.87
Na ₂ O	4.28	0.24	0.22	0.23	0.35	0.22	0.20
K ₂ O	2.31	1.38	2.04	1.34	1.92	1.40	1.84
P ₂ O ₅	0.13	1.08	1.18	1.43	1.38	1.34	1.14
S	0.03	0.14	0.23	0.16	0.15	0.19	0.18
Total	99.83	90.32	92.59	90.82	93.04	89.91	92.48

<i>b</i>		
Desert varnish		9.19% ± 0.28% H ₂ O (total)
Arizona soil		4.48% ± 0.13% H ₂ O (total)

* Sample from Fig. 1*a*, given in wt% oxide.

† Analysis is representative of feldspar-rich area below band areas 1–3.

‡ Fe computed as Fe_2O_3 ; Mn computed as MnO_2 .

§ Analyses carried out on a DuPont moisture analyser by E. K. Gibson, Jr, at NASA Johnson Space Center. Samples were heated to $1,100^\circ\text{C}$ in a flowing stream of nitrogen. Released water was trapped on a P_2O_5 cell and removed by electrolytic conductance. Sensitive to $0.1 \mu\text{g}$ of water.

caused by detrital clays, micas, feldspar quartz and so on, but which also are consistent with a possible Mn- and Fe-rich silicate mineral. We consider the present X-ray diffraction data ambiguous in view of the known heterogeneity of the scraped varnish samples.

Thin sections show that the varnish layers consist of mineral plates orientated parallel to the layering. The plates range from submicron size to a few microns long, and have perfect basal cleavage. They are strongly pleochroic with α = golden yellow, $\beta = \gamma$ = deep reddish brown ($\sim 10\text{-}\mu\text{m}$ sections). Basal sections show $2V_\alpha \approx 0^\circ$. The refractive index in all orientations is >1.795 . Microprobe analyses within large ($2\text{--}3 \mu\text{m}$) grains show them to be chemically homogeneous; however, the compositions of the grains vary from layer to layer, reflected in the analyses of Table 1. Thus, grains in dark layers are more strongly pleochroic and are more Mn-rich. We are not certain whether the grains are mixed phases (clays and oxides?) or whether they are a single silicate (unidentified) phase.

We interpret the layered botryoidal structures (Figs 1 and 2) as evidence that varnish grows vertically and laterally from nuclei in a manner similar to algal stromatolites. This conclusion is further supported by field evidence in Arizona where varnish

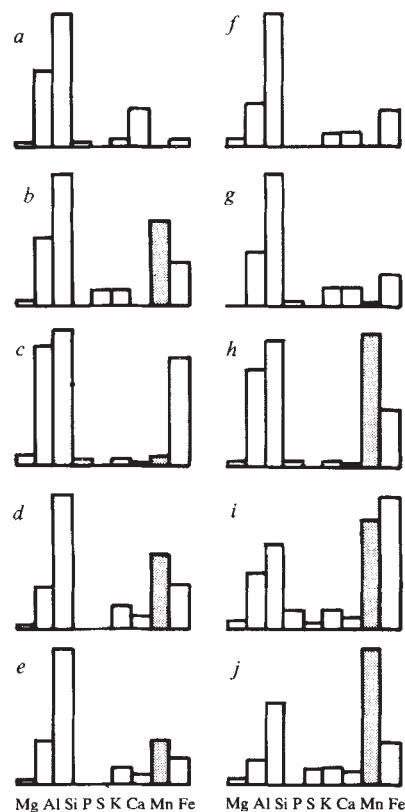


Fig. 3 SEM non-dispersive X-ray analyses (EDAX) of typical desert varnishes compared with aeolian dust, basalt substrate and a marine ferromanganese nodule. The red and black-band data* were approximated from Table 1. *a*, Arizona basalt substrate; *b*, varnish nucleation zone on Arizona basalt; *c*, Arizona varnish red band*; *d*, Idaho varnish; *e*, California varnish; *f*, Arizona soil near basalt; *g*, Arizona basalt surface outside varnish nucleation zone; *h*, Arizona varnish black band*; *i*, Utah varnish; *j*, marine manganese nodule.

is in all stages of re-covering spalls on boulders and outcrops of basalt. Old surfaces are completely covered with up to $200 \mu\text{m}$ of blue-black varnish, whereas surfaces of intermediate age show patchy coatings of lesser thickness. Field and laboratory observations indicate that freshly spalled surfaces are repopulated by microscopic black spots of varnish. Further addition of varnish occurs by enlargement of the spots which grow vertically and laterally. An analysis of one of these spots is given in Fig. 3. The spots progressively enlarge in size and number

until they coalesce and completely cover the substrate. Further thickening of the varnish occurs by continued nucleation and growth of spots (mounds) (Fig. 2). No evidence was found for the initial generation or growth of varnish as a uniform thin layer that simply increases in thickness with age.

The chemistry (Fig. 3), mineralogy and layered structure of varnish coatings from diverse localities and substrates are remarkably consistent, suggesting a common process of formation. We conclude from the lack of correlation between the composition of varnish and that of the substrate, and the presence of fine-grained dust on all sampled surfaces, that windblown dust is the source of the varnish materials. The preferential development of varnish on the tops of talus boulders is consistent with this view. This conclusion agrees with earlier suggestions¹³ and with the results of Potter and Rossman^{3,4}.

Arizona varnish has concentrated manganese by 10^2 – 10^3 (Table 1, Fig. 3) relative to airborne dust that was collected in depressions on the tops of varnished talus boulders. If varnish is nourished by dust it implies not only an effective concentration mechanism, but also an efficient mechanism for the removal of large quantities of dust from which Mn and Fe have been extracted. The removal mechanism is probably rain and dew as is suggested by accumulations of soil in drip-evaporation deposits on the undersides of boulders that are perched above soil-level on talus piles. However, neither the mechanism for manganese (and iron) concentration nor the time-scale of the deposition is known. The repeated layering in the varnish implies that manganese deposition is favoured at certain times, followed by periods when less manganese is laid down. We propose that the cyclic concentration and deposition of MnO_2 are related to environmental changes, perhaps on a seasonal or annual basis, or on a longer time-scale separating periods when climatic conditions became optimum for varnish growth.

The highly effective mechanism for concentrating manganese, and the possibility of climatic control over the cyclic banding suggests (but does not prove) that a biological process may be involved in the formation of varnish^{7,8,10,14}. The generation of varnish on rock by coalescence of growth centres is consistent with a biological process. We and J. Staley are studying the microorganisms that are associated with rock varnish. Cultures have been made in the field and in the laboratory of the surfaces and the interiors of varnishes. An assortment of bacteria, fungi

and algae consistently occur on and within the varnish coatings, including at least one *Bacillus* that oxidises Mn^{2+} to Mn^{4+} . Further work is needed to determine whether organisms produce the varnish or merely live in it.

Knowledge of the processes responsible for the formation of desert varnish coatings may be useful in understanding present and ancient weathering environments, and in dating exposed surfaces^{5,13,15}. The interpretation of optical remote-sensing data from satellites and aircraft must take into account the presence and significance of varnish in any attempt to map lithologies, as the coating locally masks the underlying rocks. If we can determine how varnish is deposited we may gain insight into the origin of remarkably similar layered deposits, namely freshwater and marine manganese nodules^{16–21}.

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Mantle $^{40}\text{Ar}/^{36}\text{Ar}$ trapped in Cretaceous deep-sea basalts

THE abundance and isotopic composition of rare gas in the mantle provides an important constraint on the origin and evolution of the Earth's atmosphere. One of sources of such information is basalts which erupted from ocean ridges. Ozima¹ stated that a high $^{40}\text{Ar}/^{36}\text{Ar}$ ratio in the mantle suggests sudden degassing at an early stage of the Earth's evolution. Several authors^{2,3} have reported excess ^{40}Ar and high $^{40}\text{Ar}/^{36}\text{Ar}$ ratios in rapidly quenched rims of young deep-sea basalts. However, the Ar composition in old ridge basalts was not known. We

report here a measurement of the isotopic composition of Ar in old deep-sea basalts. The Glomar Challenger drilled a Cretaceous ocean floor near the southern end of the Bermuda Rise in Deep Sea Drilling Project⁴. The drilled site (Site 417) is on the magnetic anomaly MO which has been estimated to be 108 Myr old.

The samples used were pillow basalts from Hole 417D, which contained only limited alteration minerals and had widespread fresh glass^{4,5}. Glassy rims of the Cretaceous pillow basalt cores, designated 22-3/24-27, 28-5/85-92, and 29-6/95-104, were investigated for rare-gas composition. (Detailed results will be published elsewhere.) The concentration and isotopic ratio of Ar are given in Table 1. The potassium content in the basaltic

Table 1 Concentration and isotopic ratio of Ar in Cretaceous deep-sea basaltic glass

Sample Core	1 22-3/24-27	2	3 28-5/85-92	4	5 29-6/95-104	6
$^{36}\text{Ar}(10^{-9} \text{ cm}^3 \text{ g}^{-1})$	1.44	2.14	1.65	4.42	0.998	5.95
$^{38}\text{Ar}/^{36}\text{Ar}$	0.194 ± 0.002	0.187 ± 0.003	0.191 ± 0.002	0.186 ± 0.002	0.188 ± 0.004	0.190 ± 0.004
$^{40}\text{Ar}/^{36}\text{Ar}$	$1,341 \pm 7$	819 ± 22	$1,103 \pm 7$	557 ± 7	$1,686 \pm 11$	567 ± 17
$^{40}\text{Ar}^*/^{36}\text{Ar}$	1,090	650	861	466	1,353	511

* Corrected for radiogenic ^{40}Ar produced in basaltic glass for 108 Myr.

glass has been determined to be 0.083, 0.091 and 0.075% for cores 22-3/24-27, 28-5/85-92 and 29-6/95-104, respectively (T. Ui, H. Haramura & H. Nagai, unpublished data). The radiogenic ^{40}Ar produced in basaltic glass during the past 108 Myr was corrected on the basis of the potassium content given above. The $^{40}\text{Ar}/^{36}\text{Ar}$ ratios corrected for radiogenic ^{40}Ar are also given in Table 1.

On the model that a given mantle component of Ar in the basalt mixed with varied amounts of contaminating atmospheric Ar, a plot of $^{40}\text{Ar}/^{36}\text{Ar}$ against $1/^{36}\text{Ar}$ gives a straight line,

$$(^{40}\text{Ar}/^{36}\text{Ar})_{\text{meas}} = S/(^{36}\text{Ar})_{\text{meas}} + (^{40}\text{Ar}/^{36}\text{Ar})_{\text{contam}} \quad (1)$$

where $^{40}\text{Ar}/^{36}\text{Ar}$ is the ratio corrected for radiogenic ^{40}Ar , and

$$S = (^{36}\text{Ar})_{\text{mantle}}[(^{40}\text{Ar}/^{36}\text{Ar})_{\text{mantle}} - (^{40}\text{Ar}/^{36}\text{Ar})_{\text{contam}}] \quad (2)$$

if the concentrations of mantle ^{36}Ar and ^{40}Ar are uniform in the samples investigated (see Fig. 1). To evaluate the amount of mantle ^{40}Ar and the $^{40}\text{Ar}/^{36}\text{Ar}$ ratio of mantle Ar trapped in the Cretaceous basaltic glass, the $^{40}\text{Ar}/^{36}\text{Ar}$ isotopic ratio for contaminating Ar and the concentration of mantle ^{36}Ar should be known.

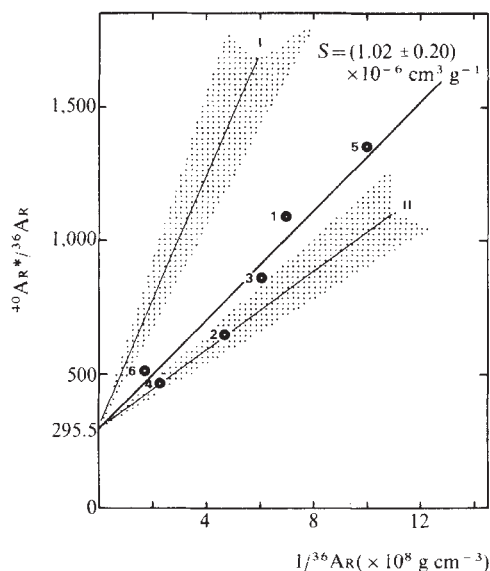


Fig. 1 A plot of $^{40}\text{Ar}^*/^{36}\text{Ar}$ against $1/^{36}\text{Ar}$. $^{40}\text{Ar}^*/^{36}\text{Ar}$ ratios are corrected for radiogenic ^{40}Ar produced in basaltic glass by decay of ^{40}K for 108 Myr. The isotopic ratio of contaminating Ar was assumed to be atmospheric (see text). For comparison, correlation lines I and II given by Dymond and Hogan⁸ are also plotted. ●, This work.

Microscopic examination of thin sections of the same cores used in this work (T. Ui, personal communication), shows that cores 22 and 29 have many cracks in the glassy rims along which alteration of glass is advanced. Gas pores have been filled with altered materials. For core 28, the alteration is so advanced that fresh glass is a minor component, and large cracks have been filled with altered materials such as carbonate, clay and zeolite. Thus the alteration in these cores is likely to be due to seawater-rock interaction at low temperature⁵. Hence the contamination of Ar can be attributed to penetration of seawater into the altered materials. Seawater which is in equilibrium with the atmosphere contains a large amount of atmospheric Ar ($10^{-4} \text{ cm}^3 \text{ STP g}^{-1}$) (ref. 6). Owing to a high diffusion rate of Ar in water, Ar in the altered materials could be replaced by Ar with the modern $^{40}\text{Ar}/^{36}\text{Ar}$ ratio.

Another possibility is contamination by seawater Ar at the time of eruption through the Ar diffusion into the quenching glassy rim of pillow basalt. In this case, $(^{40}\text{Ar}/^{36}\text{Ar})_{\text{contam}}$ may be different from that of atmospheric Ar and represent the isotopic ratio of Ar in Cretaceous seawater. However, experimental evidence for ^3He and ^{40}Ar excesses in glassy rims of modern pillow basalts^{2,3,7}, indicates that the inherent rare gases in lava were compressed tightly in the rapidly quenched rims of pillow basalts under great hydrostatic pressure and the interaction with seawater was not appreciable in the rare-gas composition. Thus we presume that the contribution from the latter process is ineffective compared with the contamination of atmospheric Ar by the penetration of modern seawater into the altered materials. A least-squares fit gives an intercept of $(^{40}\text{Ar}/^{36}\text{Ar})_{\text{contam}}$ equal to 243 ± 73 without any assumption on the isotopic ratio of contaminating Ar. This value is consistent with that for atmospheric Ar ($=295.5$) within experimental error. In the following discussion, therefore, we use the atmospheric $^{40}\text{Ar}/^{36}\text{Ar}$ ratio as the isotopic composition of contaminating Ar.

Figure 1 plots $^{40}\text{Ar}/^{36}\text{Ar}$ against $1/^{36}\text{Ar}$ with the intercept of 295.5 for the Cretaceous deep-sea basalts. It defines a correlation line the slope of which is $(1.02 \pm 0.20) \times 10^{-6} \text{ cm}^3 \text{ STP g}^{-1}$. For comparison, correlation lines for modern deep-sea basalts given by Dymond and Hogan⁸ are also plotted. As mentioned earlier, the amount of mantle ^{40}Ar and the $^{40}\text{Ar}/^{36}\text{Ar}$ ratio of mantle Ar trapped in the Cretaceous basaltic glass depend on the concentration of mantle ^{36}Ar . We use the lowest concentration of ^{36}Ar measured in sample 5 as an upper limit of mantle ^{36}Ar . From equation (2) with this value, $(^{40}\text{Ar}/^{36}\text{Ar})_{\text{contam}} = 295.5$, and $S = 1.02 \times 10^{-6} \text{ cm}^3 \text{ STP g}^{-1}$, we have

$$1.02 \times 10^{-6} < (^{40}\text{Ar})_{\text{mantle}} \leq 1.31 \times 10^{-6} \text{ cm}^3 \text{ STP g}^{-1}$$

and

$$(^{40}\text{Ar}/^{36}\text{Ar})_{\text{mantle}} \geq 1,350$$

as a lower limit of the $^{40}\text{Ar}/^{36}\text{Ar}$ ratio of mantle Ar trapped in the Cretaceous deep-sea basalts. For mantle ^{36}Ar trapped in glass of modern deep-sea basalts, Craig and Lupton⁹ have adopted $0.5 \times 10^{-9} \text{ cm}^3 \text{ STP g}^{-1}$. With this value, we have $(^{40}\text{Ar})_{\text{mantle}} = 1.17 \times 10^{-6} \text{ cm}^3 \text{ STP g}^{-1}$, and $(^{40}\text{Ar}/^{36}\text{Ar})_{\text{mantle}} = 2,340$ in the Cretaceous deep-sea basalt glass.

In conclusion, a linear correlation in the $^{40}\text{Ar}/^{36}\text{Ar}$ against $1/^{36}\text{Ar}$ plot indicates that Ar in the Cretaceous basaltic glass is a mixture between a uniform concentration of mantle Ar and varied amounts of contaminating atmospheric Ar. The $^{40}\text{Ar}/^{36}\text{Ar}$ ratio of mantle Ar trapped in the Cretaceous basaltic glass is $>1,350$, and the amount of mantle ^{40}Ar resembles that trapped in modern basaltic glass^{10,11}.

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Evolution of sulphur dioxide tolerance in perennial ryegrass

THE combustion of fossil fuels for domestic and industrial purposes, coupled with rapid industrial growth over the past 150 years, has resulted in sulphur dioxide becoming an integral part of the environment. Extensive research into the effects of sulphur dioxide on vegetation began in the 1920s, but the possibility of evolution of tolerance has only recently received attention, in spite of the implications of this for the overall effect of SO_2 in the field. We present here data that provide strong evidence for the evolution of SO_2 tolerance in a perennial ryegrass population from an urban area of Merseyside, UK.

Any environmental factor that has an adverse effect on plant growth may exert selection pressure on plant populations by acting on their inherent genetic variability, thus causing major changes in gene frequency within a few years¹. Recent research has shown the detrimental effect of SO_2 on perennial ryegrass (*Lolium perenne* L.) and the existence of substantial genetic variability in response^{2,3}. Such variation in response in the field could result in the elimination of susceptible individuals from natural ryegrass populations, thus causing an increase in the overall tolerance of these populations. Indeed, the indigenous ryegrass at Helmsshore (24 km north of Manchester) seems to have evolved tolerance to sulphur dioxide. The observations at Helmsshore, however, were based on the response of two ryegrass clones—selected as sulphur dioxide tolerant in field trials at Helmsshore—compared with many individuals of the S23 cultivar grown from seed.

To investigate overall population responses and variability in genotype response to sulphur dioxide, we have examined two long-established perennial ryegrass populations from Merseyside. These have experienced widely different ambient sulphur dioxide concentrations⁴, but the climate, soil and management conditions have been comparable, because they were collected from parklands similar in aspect, altitude and soil.

One population was collected from Newsham in the centre of Liverpool and the other from West Kirby, a rural area on the Wirral peninsula 16 km WSW of Liverpool. Records show that typical winter mean concentrations of SO_2 in the centre of Liverpool⁴ between the late 1950s and the early 1960s were 600–700 $\mu\text{g m}^{-3}$. Since that time, the winter concentrations have decreased to approximately 200 $\mu\text{g m}^{-3}$. In rural areas of the Wirral however, concentrations have been much lower, decreasing from approximately 80 $\mu\text{g m}^{-3}$ in the early 1960s to 30–40 $\mu\text{g m}^{-3}$ at present.

Table 1 Response of two perennial ryegrass populations from Merseyside to sulphur dioxide

	Control (35 $\mu\text{g m}^{-3}$)	Polluted (650 $\mu\text{g m}^{-3}$)
(a) Living dry matter		
Newsham (high SO_2 area)	0.91	0.82
West Kirby (low SO_2 area)	0.90	0.64
$P < 0.05$ LSD between populations = 0.08		
LSD between treatments = 0.14		
(b) Total (living + dead) dry matter		
Newsham	1.18	1.18
West Kirby	1.19	1.00
$P < 0.05$ LSD between populations = 0.10		
LSD between treatments = 0.19		

Values are mean shoot dry weight (g per clone). Plants were grown under a 16 h day/8 h night cycle under a light intensity of 30 W m^{-2} . Day and night temperatures were maintained at $20 \pm 1^\circ\text{C}$, and $14 \pm 1^\circ\text{C}$ respectively. Relative humidity was kept above 60%. Wind speed over the plants was 120 m min^{-1} . The results are the mean shoot dry weights of two sequential experimental runs with identical sets of clones carried out in the same fumigation conditions.

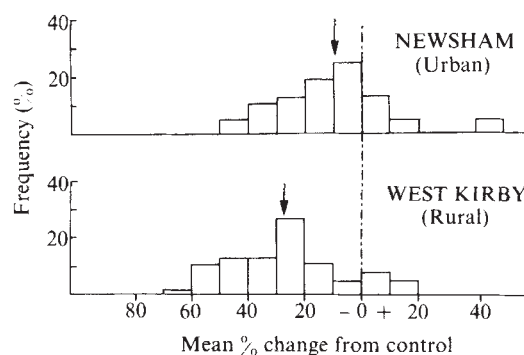


Fig. 1 Differential response to SO_2 of urban and rural ryegrass populations. Change from control measured as weight of living dry matter grown in SO_2 polluted air. Arrows mark arithmetic means of the frequency histograms.

Thirty-six individually identified clones of each population were exposed to 35 $\mu\text{g SO}_2 \text{ m}^{-3}$ (control) or 650 $\mu\text{g m}^{-3}$ (polluted) for 8 weeks in a wind-tunnel fumigation system (Table 1). In control air the mean yields of both populations were comparable. However, in the polluted air the West Kirby population showed a significant reduction in yield, whereas no significant reduction was shown by the Newsham population. Analyses of variance for both the living and total dry matter showed that there was a significant ($P < 0.01$) interaction between sulphur dioxide and the populations. Interestingly, the reduction in yield of total dry matter was less than that for living dry matter only. This suggests that the higher sulphur dioxide concentration caused an increase in the rate of senescence as well as 'invisible injury' (that is, reduction in yield without visible symptoms).

The percentage change in the living dry matter of each individual clone, as a result of the sulphur dioxide fumigation (Fig. 1), shows a wide range of genotypic variability within each population. In addition to the differential response of the populations to sulphur dioxide, the histograms reveal a reduced clonal variability within the Newsham population, presumably due to selection of the more tolerant genotypes by sulphur dioxide in the field.

These results provide strong evidence that the high sulphur dioxide concentrations prevalent in urban areas of Merseyside until the early 1960s have acted as a selective force. Furthermore, this population differentiation has persisted even though sulphur dioxide concentrations have decreased appreciably since then.

It is, therefore, important that the evolution of sulphur dioxide tolerance is taken into account when attempts are made to estimate reductions of yield in the field by extrapolation from laboratory studies. Fumigation of spaced plants in laboratory experiments may well overestimate the effects of sulphur dioxide on plant populations, as there can be no compensation for the yield reduction of sensitive individuals by the increased growth of tolerant individuals in these experiments, as would occur in dense swards. If the evolution of tolerance were sufficiently rapid in young grass swards then overall productivity would suffer only a temporary reduction, provided that the more tolerant individuals were of comparable vigour to the more susceptible. This seems likely, for in our results the yields of the two populations were similar in control treatments. Unfortunately, little is known about the rate of evolution of sulphur dioxide tolerance, although it is known that evolution of metal tolerance can occur within a few years.

In the field, plants are often exposed to mixtures of air pollutants, and the yields of some pasture grasses can be reduced severely by the synergistic interaction of sulphur dioxide and nitrogen dioxide⁵. It is thus possible that some plant populations could have evolved tolerance to combinations of air pollutants as well as to a single pollutant.

The potential for evolutionary adaptation to sulphur dioxide depends on the existence of genetically based variability in response within the population, variety or species. Such variability would be less in inbred agricultural crop varieties: also it is unlikely that substantial genetic change would occur in any crop, however variable, that was replaced annually from material bred in a low sulphur dioxide area.

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Rhizobium japonicum mutants unable to use hydrogen

FACTORS which limit nitrogen fixation by the legume nodule symbiosis may be environmental (such as illumination, water or CO₂ supply), or intrinsic (such as supply of photosynthate to the nodule¹, effectiveness of rhizobial strain). An important intrinsic feature seems to be ATP-dependent hydrogen evolution by nitrogenase, which leads to loss of energy that would otherwise be available for nitrogen fixation². Some strains of *Rhizobium japonicum* possess a unidirectional hydrogenase, capable of hydrogen oxidation but not hydrogen evolution, which recycles the hydrogen evolved by nitrogenase, regenerating ATP as well as protecting the nitrogenase from oxidative damage^{3–5}. The physiological importance of hydrogenase in this context, with its obvious implications for agriculture, has so far been deduced from studies on naturally occurring strains possessing or lacking

hydrogenase^{2,6}. A more accurate assessment requires the isolation of isogenic hydrogen uptake-negative (Hup[−]) mutants from hydrogenase-positive (Hup⁺) parents, and their comparison in physiological and agronomic studies. Such mutants would also be valuable for gene transfer and biochemical studies. We now report the isolation of Hup[−] mutants.

Our strategy was to seek a substrate for hydrogenase whose product on reduction was, or could be made, toxic. Survivors resistant to such toxicity ought to include Hup[−] phenotypes. 'Phenazine methosulphate' (*N*-methylphenazinium methyl sulphate, PMS) proved to be a good substrate for rhizobial hydrogenase⁴ and was reduced faster by bacteroid suspensions in the presence of H₂ than in its absence. In pilot experiments with *Azotobacter vinelandii*, photo-oxidation of PMS reduced in H₂ by bacteria killed some 90% of the cells; we therefore devised the procedure in Table 1, in which suspensions of largely viable⁷ bacteroids were allowed to reduce PMS and immediately treated with pure O₂; enhanced killing in the presence of H₂ was then observed. The reason for the lethal effect of oxidising reduced PMS is not clear; it may involve H₂O₂ formation⁸, a damaging effect of a free-radical derivative of PMS⁹ or localised generation of the antibiotic pyocyanine from PMS.

Survivors of the PMS-treated population were then screened by the triphenyl tetrazolium procedure reported from this laboratory¹⁰. To be confident that presumptive mutants were not random contaminants, it was necessary to label genetically the strain treated, and so a parent strain (SR) was prepared:

Table 2 H₂ evolution, C₂H₂ reduction, nodule weight and number of nodules formed by strain SR and derivative mutants of *R. japonicum*

Strain	H ₂ evolution (μmol per h per g nodule wt)	C ₂ H ₄ formed (μmol per h per g nodule wt)	Nodule weight (mg per plant)	No. of nodules per plant
SR	<0.005	73.9±2.3	193±14	27±2
SR1	24.8±2.4	54.6±3.4	162±19	23±5
SR2	18.5±1.9	46.9±2.3	243±22	27±3
SR3	24.0±2.9	56.4±7.0	217±18	22±3

Results are means ±s.e.m. for three 25-d-old Wilkin soybeans grown in Leonard jars in the greenhouse, as described elsewhere^{11,12}. At the time of planting, each seedling was inoculated with approximately 1×10⁹ cells. Nodulated roots were placed into 30-ml serum vials, the vials were sealed and incubated for 15 min at 26 °C, and 0.5-ml samples removed for H₂ quantitation by gas chromatography. After removal of the H₂ sample, 3 ml of C₂H₂ was injected into the vial, and 0.5-ml gas samples were removed at 15 min and 30 min for C₂H₄ quantification by gas chromatography.

a spontaneous streptomycin (Str, 250 μg ml^{−1})-resistant followed by kanamycin (Kan 50 μg ml^{−1})-resistant double mutant of strain USDA DES 122. This strain showed effectiveness, hydrogenase content and nodulation capacity comparable to DES 122 and retained the Kan and Str markers when re-isolated from nodules.

For PMS treatment, nodules produced by this strain were picked from 4-week-old soybean plants (cv. Wilkin) grown in Leonard jars^{11,12}, and bacteroids were obtained as described in the legend to Table 1. The conditions of PMS treatment were also as described in the Table 1 legend, except that the suspensions contained 2.4 mg bacteroids. Survivors of the PMS killing procedure were plated on to a medium previously described¹³, but also containing gluconate 5 g l^{−1}, arabinose 5 g l^{−1}, triphenyl tetrazolium chloride 0.1 g l^{−1} and streptomycin 250 μg ml^{−1}. Approximately 1.7×10⁴ colonies formed by individual survivors of the PMS treatment were screened for tetrazolium reduction. Those colonies exhibiting low dye-reduction ability after 10 d of growth were chosen and restreaked on to the tetrazolium-containing medium. The isolates consistently showing poor dye-reduction ability were each inoculated on to two soybean plants in cellophane growth pouches¹⁴. After 3 weeks of growth the roots from each pouch

Table 1 Effect of phenazine methosulphate on viability of bacteroids

Conditions	No. of survivors	% Kill
No PMS, N ₂ atmosphere	4.2×10 ⁹	—
PMS, N ₂ atmosphere	2.8×10 ⁹	23%
PMS, H ₂ atmosphere	6.0×10 ⁸	86%

A bacteroid suspension of strain USDA DES 122 from fresh soybean nodules was divided among test tubes sealed with serum stoppers and then flushed with the appropriate gas. Phenazine methosulphate (1 mM) was added, and the suspension incubated at 26 °C in the dark. After reduction of the dye (5 min), the atmosphere was replaced with O₂ and the suspension was rapidly mixed under normal laboratory illumination. Cells were centrifuged, resuspended and plated for survivor counts. The number of survivors reported is the average of two separate experiments. The bacteroid suspensions consisted of 2.0 mg dry weight bacteroids in 0.4 ml of Mg-phosphate buffer (50 mM K₂HPO₄, 2.5 mM MgCl₂, pH 7.0) and were prepared as described elsewhere¹⁵, except that the bacteroids were washed twice in the Mg-phosphate buffer.

Table 3 H₂ and O₂ uptake by bacteroids and free-living cultures of strain SR and mutant strains SR1, SR2, SR3

Strain	Bacteroids		Free-living cells	
	H ₂ uptake*	O ₂ uptake*	H ₂ uptake†	O ₂ uptake†
SR	3.7	0.70	119	230
SR1	<0.005	0.64	<0.005	276
SR2	<0.005	0.71	<0.005	240
SR3	<0.005	0.82	<0.005	253

* Expressed as $\mu\text{mol per h per mg protein}$. Nodules from 28-d-old Wilkin soybean plants were collected and the bacteroid fraction isolated in aerobic conditions as described in the legend to Table 1. Bacteroid suspensions containing approximately 1.5 mg protein per ml in Mg-phosphate buffer were assayed for uptake of H₂ and O₂ at initially saturating levels¹¹ by use of an amperometric method that used a Clark-type electrode¹⁶. Rates of O₂ uptake were determined in the absence of H₂.

† Expressed as nmol per h per 10⁸ cells. Cells were grown for 5 d on a medium designed for expression of hydrogenase¹⁰, suspended in Mg-phosphate buffer, and O₂ and H₂ uptake rates determined amperometrically. All suspensions contained 1.1–1.2 $\times 10^8$ cells per ml. Rates of O₂ uptake were determined in the absence of H₂.

were removed from the plants, and the nodulated root system was assayed for H₂ evolution by gas chromatography. Nodules lacking hydrogenase are not able to recycle H₂ produced by nitrogenase, so Hup[−] mutants would evolve H₂.

Three mutants which produced nodules evolving H₂ were obtained, SR1, SR2 and SR3; strain SR evolved no H₂ (Table 2). The three mutants did not differ greatly from the wild type in symbiotic characteristics other than the ability to use H₂ (Table 2). Nodule weight and number of nodules formed per plant for SR2 and SR3, were not appreciably different from the corresponding values for SR. Apparently, their lack of ability to use H₂ did not greatly impair their other symbiotic properties. Mutant SR1, however, showed less nodule weight than SR, SR2 and SR3. Strain SR apparently formed nodules containing greater nitrogenase activity than any of the mutants (Table 2, and other data not shown); this greater nitrogenase activity may be due to the ability of strain SR to recycle H₂. The Hup[−] phenotype of the mutants was retained on re-isolation from nodules, together with the Kan and Str markers; hence, the Hup[−] mutations were reasonably stable. Free-living cultures were grown in the conditions previously reported¹⁰ which allow expression of Hup⁺ *ex planta*, and were tested for H₂ and O₂ uptake rates by the amperometric method^{11,16}. Bacteroid suspensions were also tested. Suspensions of strain SR took up H₂ but none of the mutants did so (Table 3). Free-living cultures of the mutants as well as bacteroids isolated from nodules showed O₂ uptake rates comparable to that of strain SR (Table 3), indicating that the mutant strains still carry out normal O₂-linked respiration.

Three mutants obtained by screening 1.7 $\times 10^4$ survivors of the PMS treatment is rather a high proportion, so the possibility exists that the PMS procedure is mutagenic. We found that the PMS procedure approximately doubled the frequency of streptomycin-resistant isolates (from bacteroids of *R. japonicum* strain USDA DES 122) compared to untreated controls; some possibility therefore remains that the mutants contain additional lesions in loci other than in the H₂-uptake system. It has not so far been practicable to check by conventional reversion tests whether the Hup[−] strains are point mutations.

Greenhouse and field tests of these mutants are in progress to assess the agronomical importance of hydrogenase, and their specific biochemical defects will be investigated to aid understanding of the biochemistry and physiology of the H₂-uptake system in the *Rhizobium*–legume symbiosis.

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Kinship asymmetry in diploids

THE relatedness of one individual to another is an important variable in kin selection theory. An unusual property of relatedness is its asymmetry. For haplo-diploids, this asymmetry has been discussed¹. We show here that, surprisingly, there may also be asymmetry in relatedness between two diploids. One may in fact be more related to a person than they are to you.

The easiest way to see this is to consider a single locus, where the genotypes of two individuals are known. Possible pairs of genotypes that the individuals may have are shown in Table 1. When one individual is homozygous, and the other heterozygous with one of its alleles common to both individuals, relatedness is not reciprocal. An individual A with genotype *ww* is related to an individual B with genotype *wx* by a half, but B is related to A by one. Note that B can increase the representation of its alleles more in the next generation by aiding A than A can by aiding B.

This asymmetry in relatedness of diploids follows from the concept of relatedness as applied to cases of inbreeding². A

Table 1 Relatedness of A to B and B to A

A	Genotypes B	Relatedness	
		A to B	B to A
ww	ww	1	1
ww	wx	$\frac{1}{2}$	1
wx	ww	1	$\frac{1}{2}$
wx	wx	$\frac{1}{2}$	$\frac{1}{2}$
wx	wy	$\frac{1}{2}$	$\frac{1}{2}$
wx	yz	0	0
wx	yy	0	0
ww	yz	0	0
ww	yy	0	0

general statement of relatedness, valid for diploids and haplo-diploids alike is that the relatedness of A to B is a ratio of averages: the average, over alleles in A, of the probabilities that each will be found in a random gamete of B, divided by the average, over alleles in A, that each will be found in a random gamete of A.

In the usual kin selection analysis, one does not deal with the certainty of known genotypes, but rather with the overall expected relatedness (for example, expected frequencies of genotype pairs) that two individuals may have as the result of some pedigree relationship. Consideration of expected relatedness for diploids also gives non-reciprocal relatedness whenever there is some inbreeding and the two individuals are not identically inbred^{2,10}.

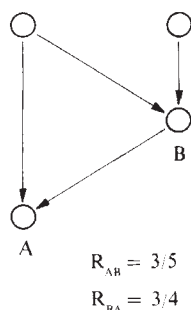


Fig. 1 Parent-offspring mating and relatedness

As it is unlikely that mating in natural populations is uniform and symmetric, non-reciprocal relatedness will be common. Simple pedigrees that have the property of non-reciprocal relatedness are brother-sister mating followed by one of the sibs outbreeding, or parent-offspring mating. A pedigree diagram for the latter case is shown in Fig. 1. Relatedness values are given as calculated from Hamilton's expression². Both of these simple pedigrees occur in natural populations; for example, they are thought likely in wolves³.

There is more asymmetry in relatedness if there is more inbreeding before the outbreeding. At the extreme limit, when inbreeding has reached essential homozygosity, offspring produced by outbreeding would be related to their inbred half-sibs by one; relatedness of the inbred individuals to their outbred half-sibs would be a half.

Although there is substantial literature on problems of calculating relatedness^{2,4,5}, non-reciprocal relatedness of diploids has escaped notice. Perhaps this is because the idea conflicts with everyday usage of the word.

Kin selection theory uses relatedness in proposing answers to two sorts of questions. One question concerns how an optimal strategist should act when faced with a choice of aiding or not aiding other individuals. The other question concerns the expected change in allelic frequency of an allele that has indirect effects on the fitness of individuals other than the allele's bearer. Non-reciprocal relatedness affects the answers to both questions but the implications for optimal strategy decisions seem the most interesting.

For haplo-diploid organisms, asymmetrical relatedness has recently been in the centre of attempts to test strategy aspects of kin selection theory⁶⁻⁸. In the diploid-diploid case, recognition of non-reciprocal relatedness might lead to testable predictions about interactions between individuals.

A major unresolved aspect of kin selection theory is the degree to which kin selection has actually resulted in effects on the evolution of phenotypes. Many aspects of behaviour and social structure have been interpreted under the kin selection paradigm. However, the theory is rather flexible, and it is easy to bend it to fit the sparse data available.

Perhaps the consequences of non-reciprocal relatedness are trivial and the magnitude or predictability of this asymmetry falls below the as yet unknown threshold for the evolution of

strategies to take it into account. It is not yet clear what magnitudes of relatedness can influence the evolution of phenotypes. Empirical studies will be necessary to resolve this. Successful empirical testing requires awareness of the subtleties of relatedness if it is to succeed in establishing the domain of validity of kin selection theory. Pending such tests, I agree with Williams⁹ that adaptation, particularly such 'fine-tuned' adaptation should not be assumed. The burden of proof lies with those who claim the adaptive significance of this or any phenomenon.

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Communal breeding in green woodhoopoes as a case for reciprocity

RECIPROCITY¹, or reciprocal altruism², and kin selection³ are concepts basic to sociobiology. Although both theories have been widely accepted⁴, little critical evidence exists for either^{5,6}. Communal, or cooperatively breeding, birds are important organisms for testing these theories because nonbreeding helpers regularly provide aid to nestlings, such as food and protection. Helpers are often related to the nestlings they aid, frequently being older siblings^{7,8}, leading some authors to conclude that kin selection is the evolutionary basis for the apparent altruism seen in cooperative breeders^{8,9}. However, the possible influence of other, individual strategies has not been completely separated from kin selection for any communal bird; this would require elimination of kinship ties as a variable⁵. Aid by a helper to an unrelated young bird must either be considered an 'accident' or it suggests that factors other than, or in addition to, kin selection might be the evolutionary bases for helping behaviour, at least in that particular species. Here we report information on a tropical, communal bird, the green woodhoopoe (*Phoeniculus purpureus*), that suggests helping is a strategy for personal gain¹⁰.

We have studied the social behaviour of green woodhoopoes near Naivasha, Kenya, from July 1975 to the present. During the first period of field work (July 1975-May 1976), we individually marked almost all members of 24 flocks, some 148 birds. Subsequently, we have marked an additional 109 woodhoopoes, most of which were juveniles produced in these flocks. We have also recorded and marked immigrants into, and emigrants from, the original groups. Agonistic behaviour is common enough to permit us to assign dominance and subordinate status to most flock members. Thus, kinship ties and social statuses of many flock members are now known.

Social units or flocks of green woodhoopoes consist of 2-16 birds and contain a single breeding pair. Other flock members, usually relatives of one or both breeders, serve as nest helpers. When a flock is decimated, or when a new flock forms, 1-4 females merge with 1 or 2 males; sometimes these social units consist of individuals unrelated to either potential breeder. Unrelated birds of the same sex were usually females¹¹.

Woodhoopoes of all ages, sex and social classes disappear at a rate of about 40% per year (January 1976-January 1978); we

equate most disappearances with mortality¹². Because of this high mortality, we have obtained information in only three instances concerning the roles of unrelated birds in subsequent breeding activities.

First, in early June 1977 the L-h flock consisted of a single adult male, GL, banded in this territory in late 1975, and four females not present the previous January. Two females, GR and WR, were banded together as immatures in an adjacent territory in August 1975, and were almost certainly siblings. Two younger females, OL and RL, from outside the study area and thus not closely related to GR and WR, were also present. Male GL and female GR bred in early June, with the other three females behaving as nest helpers. RL and OL, unrelated to the nestling, provided more food than did the sibling (WR) of the female breeder (Table 1). Thus, differences in quantity of food delivered to this nest were apparently unrelated to previous foraging experience¹³.

Food deliveries by RL and OL increased conspicuously as the nestling matured (Fig. 1). (The increased activity of these helpers was probably not due to increased needs of the single nestling as it grew. During the same period an unaided pair successfully reared two healthy young.) These two birds apparently sought to interact directly with the nestling rather than to deliver food to the female parent, GR. By 13 July, GR remained outside the nest cavity for extended periods, providing the opportunity for helpers RL and OL to enter the nest and feed the nestling directly; from then on their contributions increased proportionately more than did those of the male parent. By 24 July, the nestling perched at the cavity entrance and these helpers groomed and vocalised to the young bird, in addition to feeding it.

Second, in June 1977, the H5 flock consisted of an adult male, OL, his younger male sibling, WR, the adult breeding female, YL, banded in this territory in 1975, a juvenile female offspring, GL, of YL and her previous mate, and a new subadult immigrant female, WL, unrelated to the others. YL soon disappeared and WR returned to his previous territory. Male OL and female WL began courtship feeding and copulation. WL now also occasionally fed GL, then almost a year old and long self-sufficient, and initiated vocal rallies with her. Vocal rallies are an important intra-group behaviour promoting cohesion. In short, WL seemed to develop a closer personal bond with GL. When WL nested in early July, GL became an active nest helper (Table 1), although she was unrelated to either breeder. Female WL, a newcomer to the H5 territory and unrelated to the original breeding pair, thus inherited ownership of a territory, breeding

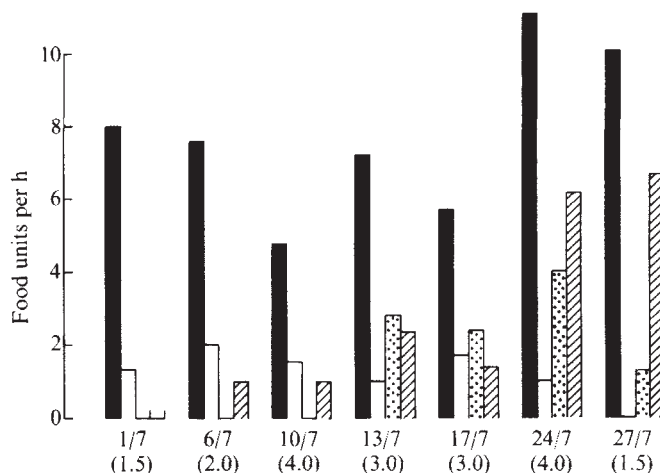


Fig. 1 Food contributions of L-h flock members after the single nestling had hatched. Food units are based on size categories; large, medium and small, multiplied by feeding visits. Numbers on the horizontal axis indicate date and in parentheses, the number of hours observation on each date. Flock members are: ■, GL, male parent; □, WR, female sibling of female parent; ▨, RL, and ▩, OL, females unrelated to either parent. OL was not more than 1 yr old. On 13 July the female parent first remained outside the nest for extended periods. From this date on, contributions of RL and OL, in particular, increased proportionately more than did those of the male parent.

status and a nest helper, GL, within a year or less of her arrival.

Third, in early September 1975, the AD flock consisted of an incubating female and two males. A single young hatched on 17 September. Two females (GR and YR) from an adjacent flock occasionally foraged with the males in the latter's territory as the female breeder incubated, but usually they remained in their own flock, where they helped feed recently fledged juveniles. These females did not visit the AD nest until after the female parent had disappeared, between 7 and 13 October. By the latter date both had joined the AD males, with GR assuming the role of foster mother.

The apparently conflicting interests of the four adults at this nest led to a great variation in feeding patterns. Female GR behaved in typical maternal fashion, often remaining in or near the nest as other flock members foraged elsewhere. When a bird approached the nest with food, GR quickly entered the nest to intercept the food item, presumably passing it on to the nestling.

Table 1 Activities of helpers at two green woodhoopoe nests in 1977

Flock: helpers	Incubation			Stage of nesting cycle			
	Food units	Feeding visits	Non-feeding visits	Nestling		Non-feeding visits	Direct interaction with nestlings*
				Food units	Feeding visits		
L-h: (17)				(19)			
♂ GL†	96	45	0	144	71	3	Ca. 12 ¶
♀ WR	7	4	17	24	13	15	5¶
♀ RL	10	6	3	33	18	4	9¶
♀ OL	2	2	5	52	30	1	9¶
H5: (13)				(16)			
♂ OL†	102	54	2	159	89	1	1
♀ GL	32	16	7	114‡	64	6	8
♂ WR	0	0	0	33§	15	0	2

Food units are based on size categories of food brought to the nest: large (3), medium (2), small (1) × feeding visits. Numbers in parentheses indicate hours of observation.

* Includes entering the nest, vocalising directly into the nest, or grooming the nestling at the cavity entrance.

† Male breeders provided significantly more food both to the incubating female and to the nestling(s) than did the helpers (Mann-Whitney *U*).

‡ GL brought significantly more food to the nest than did WR (Mann-Whitney *U* = 21); GL is unrelated to the nestlings, whereas WR is an uncle.

§ Male WR returned to this territory at about the time the eggs hatched and subsequently fed the young.

|| Male began to feed young directly when it came to nest entrance. Would not pass food to females other than GR, although several females attempted to take it.

¶ After 10 July both feeding and other interactions increased for the L-h helpers, when the female parent remained outside the nest for extended periods (see Fig. 1).

Female YR almost always gave food to GR, apparently to avoid aggression by the latter. Helper male WL strongly preferred to feed the nestling directly, but GR frequently thwarted him. For example, during a 4-h watch on 16 October, WL attempted 10 times to go directly to the nestling, but was intercepted each time by GR. The male breeder, GL, also frequently attempted to feed the nestling directly, but was equally likely to pass the food to his new mate, GR. GR seemed to behave in a manner designed to condition the males to accept her as the new breeder and to maximise her bonds with the unrelated nestling. The nestling fledged on 22 October and was cared for by all four adults until its disappearance between 13 and 18 November.

Our observations of 13 other flocks, where helpers were either related to the nestlings or where relationships were unknown, also support the suggestion that helpers vie for the opportunity to feed young. We have recorded 69 instances in which one helper has either stolen or begged food from another and then delivered it to the nestlings, and 38 cases where a helper actively avoided the soliciting female parent, to feed the young directly. These apparently disorganised behaviours at the nest make sense if helpers seek to establish personal ties with nestlings, as we suggest.

The data presented here suggest that nestlings, related or not, are valuable resources and that potentially it is to a woodhoopoe's advantage to feed and interact with them (helpers preen and vocalise vigorously to older nestlings), because the helper's subsequent attainment of breeding status and breeding success often depends on the presence of subordinate flock mates (our unpublished work); that is, nestlings are a potential resource for helpers.

In 1978 the contributions of female helpers GL and OL of the H5 and L-h flocks, respectively, were greater than in 1977, even though food was probably less plentiful. This second year of data strengthens the conclusion that such behaviour is adaptive, rather than aberrant.

The cases described here do not support an interpretation of helping behaviour relying on kin-selected altruism. The L-h and H5 flocks each contained a helper closely related to one of the breeders, as well as one or two helpers not related to either. At both nests, young received more food and other attention from unrelated helpers than from relatives. Thus, although most woodhoopoe flocks consist of relatives, these cases suggest that kinship ties (beyond parent-young) and helping behaviour in green woodhoopoes may not be causally related; instead, individual woodhoopoes can apparently gain by helping any young, at least in part because those young might later enhance the helper's future reproductive success.

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Effect of agrocin 84 on attachment of *Agrobacterium tumefaciens* to cultured tobacco cells

CROWN GALL DISEASE of dicotyledenous plants has aroused considerable interest, partly because of its economic consequences, and partly because it is the only known example of a bacterially induced cancer. The causative organisms are various strains of *Agrobacterium tumefaciens* harbouring large plasmids¹⁻³. Parts of these plasmids are transmitted to plant host cells, where they are maintained and transcribed^{4,5}. In horticultural conditions, biological control of the disease can be achieved by inoculating susceptible plants with a non-pathogen, *A. tumefaciens* K84 (refs 6, 7) and is due to the production of an intraspecific antibiotic, agrocin 84, by this strain⁸. Although the structure of agrocin 84 has been partially elucidated⁹, the mechanism by which it prevents induction of crown gall is poorly understood. We report here the transformation of cultured tobacco cells to crown gall tumour cells and present evidence, using this *in vitro* transformation system, that agrocin 84 blocks the initial step of tumour induction, that is, the attachment of pathogen to host.

Table 1 Effect of agrocin 84 on *in vitro* transformation of habituated tobacco cells by *A. tumefaciens*

Strain	Nopaline content of extracts		Tumour development on tobacco plants
	Callus tissue (% dry wt)	Cell medium ($\mu\text{g ml}^{-1}$)	
C58	0.35	<0.04	+
C58	<0.01	<0.04	-
(agrocin-treated)			
C58 (NT-1)	<0.01	<0.04	-
C58(NT-1)	<0.01	<0.04	-
(agrocin-treated)			

A. tumefaciens C58 and C58 (NT-1) were incubated for 12 h in peptone broth (0.4% Bacto peptone, 0.05% MgSO_4 , 2% sucrose) and the logarithmically growing cells were collected by centrifugation at 5,000g for 15 min at 25 °C. After resuspension in MM growth medium²³ both strains were incubated in the presence or absence of purified agrocin 84 (unpublished data) for 2 h (12 unit ml^{-1} , where 1 unit ml^{-1} is the quantity required to reduce the first order growth rate constant by 50% under defined growth conditions). Aliquots of the four bacterial suspensions were inoculated onto wounded tobacco plants, embedded for electron microscopy and introduced into cultures of habituated tobacco cells maintained in Filner growth medium²⁴ and incubated at 25 °C in an orbital shaker. These tobacco cultures were incubated for 4 weeks, rifampicin (15 $\mu\text{g ml}^{-1}$) being added 48 h after infection. Plant cells were separated from growth medium and both were extracted for amino acids essentially as described by Bomhoff²⁵. Guanido compounds were detected and estimated by modified versions of the Sakaguchi method^{26,27}. A component of the aqueous extract from callus tissue infected with *A. tumefaciens* C58 was identified as nopaline by electrophoresis at pH 3.5 and paper chromatography²⁸ using authentic samples of arginine, octopine and nopaline as references.

A. tumefaciens C58 is a member of the large group of pathogens harbouring the nopaline-type tumorigenic (Ti) plasmids. In addition to coding for enzymes catalysing nopaline synthesis in transformed plant cells and nopaline hydrolysis in the bacteria, these plasmids code for components which render the bacteria sensitive to agrocin 84^{2,8,10-13}. When strain C58 is exposed to agrocin 84, a few bacteria develop resistance to the antibiotic. They are avirulent⁸ and resemble the 'heat-cured' derivative of strain C58 (named C58(NT-1)), in that they do not carry a Ti plasmid².

Many authors have suggested that the first step in the tumour induction process involves a specific interaction of a surface component of the bacterial envelope with wound-exposed



Fig. 1 Scanning electron micrographs of *A. tumefaciens* C58 attached to the surface of habituated tobacco callus cells, 4 h after infection. Magnification of *a*, *b* and *c*, $\times 1,740$, $\times 34,000$ and $\times 35,800$ respectively. In *c* the pathogenic bacteria were preincubated with agrocin 84 for 2 h before inoculation into the tobacco cultures.

portions of the host cell wall¹⁴⁻¹⁶. Moreover, the correlation between pathogenicity and agrocin 84 sensitivity in *A. tumefaciens* C58 and other nopaline-utilising strains has led to the suggestion that surface receptors which facilitate the adherence of these bacteria to the host cell may also bind agrocin 84 (refs 13, 17). The early events of tumour induction are difficult to study using wounded plant tissue. Thus, by using *A. tumefaciens* C58 and its heat-cured, plasmid-free derivative, strain C58 (NT-1), to infect suspension cultures of habituated tobacco cells, we have developed an *in vitro* transformation system, amenable to a detailed investigation of the initial stages of tumorigenesis and of the role of agrocin 84 in preventing tumorigenesis.

The results of preliminary transformation experiments reported in Table 1 clearly indicate that the *in vivo* and *in vitro* transformation mechanisms are similar. The cultures of strain C58 which induced tumour formation on wounded tobacco plants also induced nopaline synthesis in habituated tobacco cells and both processes were blocked by the action of agrocin 84. Cultures of the heat-cured derivative, strain C58(NT-1), were not able to induce either *in vivo* or *in vitro* transformation. The concentration of nopaline found in C58 transformed tobacco callus cells was similar to that found in excised tumour tissue (J. Firmin, personal communication) and within the limits of detection ($0.04 \mu\text{g ml}^{-1}$), nopaline was not found in the incubation medium. This finding is perhaps significant in relation to the mechanism of transfer of tumorigenic DNA to non-pathogenic recipients: although octopine is known to increase the frequency of plasmid transfer in octopine-utilising strains of *A. tumefaciens*, nopaline does not enhance the *in vivo* frequency of plasmid transfer in a number of nopaline-utilising strains, including strain C58 (ref. 18).

The idea that *A. tumefaciens* attaches to specific sites in wounded plant tissue originated from the observation that tumour formation could be inhibited by the inclusion of avirulent strains of *A. tumefaciens* in inoculums of virulent strains¹⁹. If this is the case, then pathogenic and non-pathogenic strains would possess similar affinities for these sites. On the other hand, if an agrocin 84 receptor participates in the attachment of pathogen to host, then a marked difference in the binding properties of pathogenic and non-pathogenic strains would be expected. The *in vitro* transformation system permitted an investigation of these possibilities.

The infection experiments presented in Table 2 were carried out in conditions similar to those used for transformation, but in order to quantify the percentage of free to bound bacteria after a short infection period (4 h) strains C58 and C58(NT-1) were radiolabelled with ^{32}P before agrocin 84 treatment and the subsequent addition of the bacteria to suspension cultures of the plant host. The results obtained do not agree with the hypothesis that pathogenic and non-pathogenic strains have equal affinity

for the host, but suggest that the surface properties of pathogenic and non-pathogenic strains of *A. tumefaciens* are different. Whereas over 90% of the pathogenic C58 strain remained associated with the tobacco cells after fractionation, only 15% of the non-pathogenic derivative C58(NT-1) was located there. Furthermore, pretreatment with agrocin 84 consistently reduced the affinity of strain C58 for its host by a factor of two to three, but did not significantly alter the binding capacity of strain C58 (NT-1). The observations made when the infected tobacco cells were examined under the scanning electron microscope corroborated our interpretation of the results of the binding experiments reported above and further, dramatically demonstrated that the formation of a specific infection complex is essential for tumorigenesis. This cytological study revealed that the rod-shaped, non-pathogenic bacteria of strain C58(NT-1) lay recumbent on the surfaces of the tobacco cells, randomly and sparsely distributed. Pretreatment with agrocin 84 had no apparent effect on the morphology or distribution of

Table 2 Comparison of the affinities of strains C58 and C58(NT-1) for habituated tobacco cells after preincubation in the presence or absence of agrocin 84

Strain	Applied bacteria associated with tobacco cells (%)	Average no. bacteria per tobacco cell ($\times 10^{-4}$)
C58	93	14.5
C58		
(agrocin-treated)	43.8	6.08
C58(NT-1)	15.5	2.96
C58 (NT-1)		
(agrocin-treated)	12.6	2.90

A. tumefaciens strain C58 and C58 (NT-1) were incubated in peptone broth for 12 h in the presence of ^{32}P ($5 \mu\text{Ci ml}^{-1}$). The cells were collected and washed 4 times in iso-osmotic phosphate buffer, pH 7.4 ($0.155\text{M NaH}_2\text{PO}_4$ adjusted to pH 7.4 with $0.103\text{M Na}_2\text{HPO}_4$). Each strain was then incubated in the presence and absence of agrocin 84 and inoculated into suspension cultures of habituated tobacco cells (see legend to Table 1). After 4 h aliquots of the infected tobacco cultures were layered over Ficoll (15% w/v in phosphate buffer) and centrifuged at $1,800g$ for 1 h. In such conditions 'free' bacteria are pelleted and the tobacco cells remain packed at the Ficoll interface. Approximately the same values were obtained in separate experiments in which separation of plant and bacterial cells was achieved by filtration through siliconised glass fibre filter paper disks. Controls utilised labelled bacteria incubated in plant growth medium in the absence of tobacco cells. Tobacco cells were stained with Trypan blue and counted using a haemocytometer. The specific activities of the bacteria were monitored from turbidity measurements and radioactivity determinations after Millipore filtration.

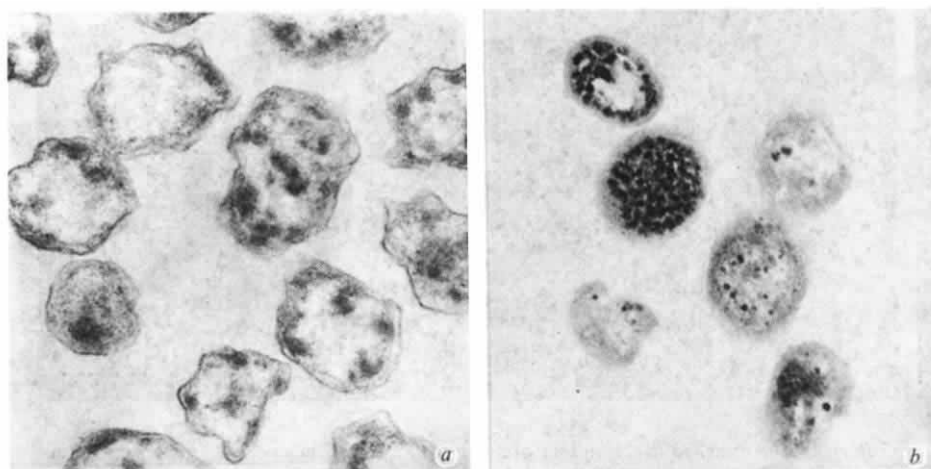


Fig. 2 Electron micrographs of transverse sections through *A. tumefaciens* C58 bacteria. *a*, Normal pathogenic C58 cells, $\times 2,780$; *b*, C58 cells after a 2 h exposure to agrocin 84, $\times 2,780$. The bacteria were prefixed in glutaraldehyde (2% v/v), post-fixed in osmium tetroxide (1% w/v) and embedded in Epon-araldite. Sections were stained with uranyl acetate followed by lead citrate.

these bacteria. In striking contrast however, the pathogenic C58 bacteria exhibited a marked tendency to cluster together and isolated cells were rarely observed. The bacteria did not apparently cluster around lesions in the host cell wall, but in some cases the entire surface appeared to be enveloped by the bacteria and in others localised areas were densely populated (Fig. 1*a*). Furthermore, the rod-shaped cells were orientated such that one end was embedded in the plant cell surface (Fig. 1*b*) and often a halo of diffuse material was observed around these bacteria. We do not consider that the observed polar attachment of bacteria to host is an artefact of the dehydration and fixation procedures as a previous cytological study of plants infected *in vivo* with *A. tumefaciens* showed the same phenomenon¹⁴ and this is the mode of attachment of members of the closely related genus *Rhizobium*²⁰ to the tips of root hairs of their plant hosts²¹.

The effect of agrocin 84 on strain C58 was immediately obvious; clustering was still apparent, but the majority of the bacteria were spherical and paired (Fig. 1*c*). As agrocin 84 is bacteriostatic (data not shown), the observation that the agrocin 84 treated cells were often located in pairs on the surfaces of the host suggested that bacteria which had entered the division cycle were unable to complete the process after the addition of agrocin 84. The further observation that these cells had rounded up suggested that agrocin 84 may act by disrupting the integrity of the cell wall.

To compare the anatomical features of normal and agrocin-treated bacteria, thin sections of these cells were examined under the transmission electron microscope. The electron micrographs shown in Fig. 2 are representative of our observations: untreated pathogenic cells of strain C58 were indistinguishable from cells of strain C58(NT-1) preincubated in the presence or absence of agrocin 84 and in all cases the cell envelopes were clearly defined. In contrast, the envelopes of agrocin-treated C58 cells were, when present, ill-defined and fragmented. Additionally, only these agrocin-treated cells contained large, electron dense granules and thus we suggest that agrocin 84 interferes with the synthesis of the cell wall rather than acting on preformed structures and that the granules may represent a build up of cell wall precursors.

The data presented here support the hypothesis that the cell surfaces of strain C58 and C58(NT-1) are different and that this difference is due to the presence of the tumorigenic plasmid (data not shown and ref. 2). The Ti plasmid of strain C58 seems to code for enzymes involved in the synthesis of surface components, believed to be capsular lipopolysaccharides²², which mediate the specific attachment of bacterium to host. It is possible that these same components are also directly responsible for sensitivity to agrocin 84, but we consider this unlikely. Sensitivity to agrocin 84 is strictly dependent on the nutritional status of strain C58 and growth media which promote the synthesis of capsular lipopolysaccharide completely inhibit

agrocin 84 sensitivity (data not shown). Furthermore, amongst the nopaline-utilising strains of *A. tumefaciens*, some pathogens are insensitive to agrocin 84 and conversely, some non-pathogens retain sensitivity (data not shown and ref. 13). Thus the correlation between pathogenicity and agrocin 84 sensitivity may be fortuitous rather than direct. We propose that agrocin 84 prevents tumorigenesis by causing the fragmentation or loss of the cell envelope and thereby impairing the ability of the bacterium to transfer tumorigenic DNA to its host. As agrocin 84 sensitivity is plasmid-coded in strain C58, the subsequent loss of this plasmid from cells incubated in the presence of this antibiotic is a requirement for survival.

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Neutralisation of respiratory syncytial virus by cat serum

RESPIRATORY SYNCYTIAL (RS) virus has been identified as a major cause of lower respiratory infection in infants, and the epidemiology of RS virus infection is characterised uniquely by annual winter outbreaks in the infant population and by antigenic stability of the virus¹. The initial isolation of RS virus was from a captive chimpanzee² and more recently an antigenically similar virus has been associated with respiratory disease in cattle³⁻⁵. The bovine and human RS viruses, although antigenically similar, can be differentiated by their different host range *in vitro*⁶. The bovine RS virus had a wider host range, multiplying in bovine, hamster, swine and primate cells, whereas the human RS virus multiplied only in bovine and primate cells. We have observed recently, however, that human RS virus (but not bovine RS virus) can multiply efficiently in secondary cultures of feline embryo cells. The cytopathology induced by RS virus infection of feline embryo cells was similar to that described previously for African green monkey (BS-C-1) cells⁷: the surfaces of infected cells were seen to be covered by long slender filaments when viewed by scanning electron microscopy or by immunofluorescent staining (C.R.P. and J. E. Parry, unpublished data). The susceptibility of feline embryo cells to RS virus, and an observation that the sera of some laboratory cats in the USA inhibited RS virus (R.M. Chanock, personal communication), suggested that cats might be susceptible to RS virus infection. We therefore examined a random sample of cat sera, and we show that antibody to RS virus is widespread in domestic cats in Britain.

Fig. 1 Histogram of RS virus-neutralising activity in normal and SPF cats. Approximately 10^4 plaque-forming units of the RSN-2 strain of RS virus were mixed with twofold serial dilutions of each serum, and incubated at 4 °C for 18 h. The infectivity remaining in each tube was assayed by titration on BS-C-1 monolayers. The percentage reduction of infectivity was determined by plaque counting after a 7-d incubation at 31 °C under 0.9% (w/v) agar overlay and plotted against serum dilution. The dilution producing a 50% reduction of infectivity was determined graphically for each serum.

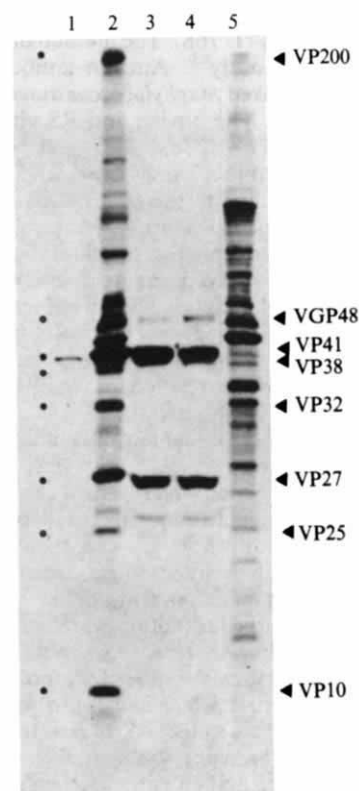
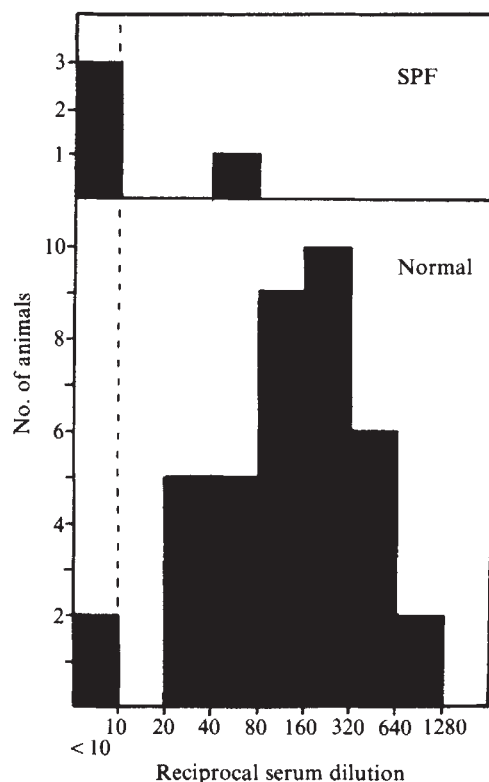


Fig. 2 Autoradiogram showing virus polypeptides precipitated by feline and bovine sera. Serum was incubated with ^{35}S -labelled RS virus-infected cells or a polyethyleneglycol (PEG) extract prepared from them for 1 h at 37 °C. Antibody-antigen complexes were precipitated with formalin-fixed heat-killed *S. aureus*¹⁰. Washed *S. aureus* precipitates were incubated at low pH to break antigen-antibody bonds. Viral antigens released into the supernatant were run on SDS-polyacrylamide gels (5.5–12% acrylamide gradients). Track 1, immunoprecipitate, cat serum + PEG extract of RS virus-infected cells, showing VP41. This band was absent when uninfected cells were used. Track 2, control. Radiolabelled RS virus-infected cells. Track 3, immunoprecipitate, bovine serum + PEG extract of RS virus-infected cells. Track 4, immunoprecipitate, bovine serum + RS virus-infected cell pellet. Track 5, control, radiolabelled uninfected cells.

Thirty-nine cat sera were obtained, representing a random selection of animals ranging from 2 months to 10 years old, 19 of which had been collected in 1975–6 and 20 in 1977–8. Four sera from specific pathogen-free (SPF) animals were also examined. The neutralising activity of these 43 sera was assayed as described in the legend to Fig. 1, and the 50% neutralisation end-points were determined graphically by plotting percentage recovery against serum dilution. The RSN-2 strain of RS virus⁷ was used as the test virus. The results are presented in Fig. 1. Thirty-seven of the thirty-nine sera from normal cats had neutralising activity, with a geometric mean titre of 1/147, and only two were negative ($<1/10$). Three of the four sera from SPF cats, on the other hand, were negative ($<1/10$) and the one positive serum had a titre of 1/50 (approximately threefold less than the mean titre of the normal group). These results show clearly that the majority of the sera of domestic cats in this sample were able to neutralise the infectivity of RS virus. There was no obvious correlation between the level of neutralising activity and age of the animals, and the titres of the sera collected in 1975–6 and 1977–8 were distributed similarly. The greater frequency of negative sera from SPF animals (0.75 compared with 0.05 for normal animals) suggests that the neutralising activity represented an immune response.

This conclusion was supported by the result of an experiment in which extracts of ^{35}S -methionine-labelled infected and uninfected BS-C-1 cells were mixed with either a high titre convalescent bovine anti-RS virus serum, or one of the cat sera with

high neutralising activity (1/768). The method of radiolabelling was as described previously^{8,9}. Antigen-antibody complexes were precipitated with fixed *Staphylococcus aureus* as described in the legend to Fig. 2. The bovine anti-RS virus serum precipitated VGP48, VP41, VP38, VP27 and VP25, but not VP200, VP32 or VP10 (tracks 3 and 4 in Fig. 2). Significantly, no polypeptides corresponding to the high molecular weight glycopolypeptides described as RS virus-specific by Levine¹¹ were observed, confirming our previous interpretation^{8,9} that these polypeptides were host-derived. Figure 2, track 1, shows that the cat serum possessed antibody able to combine with a viral protein which electrophoresed with the mobility of VP41, the nucleoprotein of RS virus^{8,9}. No corresponding band was observed when the serum was reacted with uninfected cells (data not shown).

These observations suggest that most, if not all, domestic cats in this locality have experienced RS virus infection. The presence of neutralising activity in the sera of cats of all ages (and in one of the four SPF animals) suggests that cats may be highly susceptible to RS virus infection, and that they are exposed to infection early in life. It remains to be determined whether illness is associated with infection, and whether the virus inducing the immune response is transmitted horizontally among cats. It is possible that a feline RS virus exists, but as yet none has been isolated. The relevance of these observations to the human disease pattern is a question which merits further investigation.

We have not been able to detect RS virus antibody, however, by indirect immunofluorescent staining of acetone-fixed RS virus-infected cells using pooled and absorbed cat sera and an FITC-conjugated rabbit anti-cat globulin (Nordic). It would appear that the immunoprecipitation of radiolabelled viral protein is the more sensitive method for detection of RS virus antibody in cat serum.

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HLA-DR specific human suppressor lymphocytes generated by repeated *in vitro* sensitisation against allogeneic cells

THE HLA complex is composed of a set of genes closely linked on chromosome 6, which may have important functions in the regulation of the immune response. The HLA-A, -B and -C loci control serologically defined antigens present on most cells. The HLA-D region controls two groups of determinants: (1) HLA-DR (DRw1 to DRw8) antigens, restricted to B lymphocytes,

Table 1 Decrease in the specific proliferative response of the twice-primed PLT cells (PLT II)

Responding PLTs: anti DRw1 PLT I (c.p.m.)*	PLT II (c.p.m.)*	Restimulating cells No.	HLA-DR	% Suppression
620	1,268	1	2/- NC	—
24,678	17,768	2	2/1 SC	.29
15,861	8,542	3	2/1	.47
21,626	16,267	4	1/1	.25
15,383	10,939	5	1/5	.29
19,528	11,257	6	2/1	.43
8,224	9,781	7	7/7	—
6,316	5,790	8	5/7	.9
6,049	7,628	9	2/7	—
12,111	14,763	10	7/3	—
8,728	10,194	11	5/-	—

PLT I (anti DRw1) was produced within a normal family by sensitising *in vitro* 10×10^6 lymphocytes (isolated by Ficoll-Isopaque gradient) from the father DER. F (DRw2/-) with 10×10^6 2,500 rad γ -irradiated cells from his child DER. C (DRw2/DRw1) in 20 ml RPMI 1640 with 20% normal pooled human plasma and antibiotics (100 mg ml⁻¹ streptomycin, 100 IU ml⁻¹ penicillin), in plastic culture vials (Falcon 3013). They were incubated at 37°C in a 5% CO₂ air atmosphere for 10 d. PLT II was produced by sensitising a second time *in vitro* 10×10^6 PLT I cells with 10×10^6 irradiated cells from the same stimulator over 7 d. PLT testing was carried out in V-bottom plastic culture microtrays: 5×10^4 responders in 0.05 ml and $5 \cdot 10^4$ stimulators in 0.1 ml were added in each well. 48 h later cultures were pulse-labelled for 18 h using ³H-thymidine (2 μ Ci per well) (specific activity, 1 Ci mmol⁻¹) and counted in a liquid scintillator. HLA-DR antigens were determined serologically and by PLT testing. Per cent suppression was calculated using the formula:

$$\% \text{ Suppression} = \frac{1 - \text{test c.p.m. median value}}{\text{control c.p.m. median value}} \times 100$$

* Median values of three cultures.

† NC, negative control; SC, specific control.

detected by serology and equivalent to the I region-associated antigens (Ia) of the mouse; (2) HLA-D stimulating products (Dw1 to Dw11) defined by mixed lymphocyte reactions (MLR). Sensitisation of human lymphocytes with irradiated allogeneic stimulatory cells *in vitro* produces primed lymphocytes¹⁻⁵. These *in vitro* primed lymphocytes develop an accelerated secondary proliferative response when restimulated by the specific priming cell or by any cell possessing HLA-D region products identical to the priming cells. Familial and population studies^{6,7} show this secondary proliferation (MLR II or PLT) to be more correlated to HLA-DR antigens serologically detected than to D specificities defined by HLA-D typing (MLR I). It has also been suggested that, within informative families bearing haplotypes with unusual associated D-DR products, DR antigens and HLA-D stimulating products could be distinct determinants⁷. However, absolute proof—a recombination between these two products—is still lacking. The accelerated proliferative capacity of primed lymphocytes represents a function which depends on the allogeneic contact; we have investigated the possible induction of suppressor cells by repeated *in vitro* sensitisation. We show that lymphocytes sensitised twice '*in vitro*' behave as active suppressors of the allogeneic response and are specific for the HLA-DR antigens presented by the stimulator lymphocytes.

The first indication that a suppressive activity can be detected in secondary cultures comes from the observation that twice-sensitised PLT cells (PLT II) give a lower accelerated proliferative response when restimulated by specific cells than classical PLT sensitised only once (PLT I). The kinetic study of this secondary proliferation has clearly shown that the proliferation was decreased and not delayed (data not shown). The proliferative response of PLT DER AC (PLT I) (an intrafamilial PLT generated against a DRw1-bearing haplotype) was compared with the response of the same PLT sensitised twice (PLT II) by the same priming cell (Table 1). The proliferative response of PLT I (anti DRw1) was significantly higher than the response of PLT II against DRw1-positive cells. As expected, both PLTs give the same low response against DRw1-negative cells. These results suggest that the lower specific response obtained with the twice-primed PLT (PLT II) is due to (1) depletion of the specific

responding cells, or (2) to the generation of active suppressor cells after multiple *in vitro* restimulations. The latter hypothesis has been tested in a three-cell assay.

The three-cell assay is a proliferative assay; the three co-cultured cells were unprimed responding cells, irradiated stimulating cells and possible suppressor cells (responder cells primed twice and γ irradiated). The proliferative responses in the presence or absence of the suppressor cells were evaluated and compared on day 7 of culture. The results of experiments of this kind are shown in Tables 2–5. Addition of twice-primed irradiated cells (PLT II) in a three-cell assay induced an active suppression in some combinations. The specificity of this suppression was analysed using well typed unrelated and informative familial cells. It was first observed that a PLT II anti-DRw7 in family DEG (Table 2) was only suppressive when added in the combinations where the stimulating cells possess 'a' haplotype which codes for the DRw7 antigen. In the same experiment the response against an unrelated stimulating cell, GIL, DRw7/DRw7, was also suppressed. The specificity of the suppression in this and other families (data not shown) seemed to segregate with an HLA haplotype.

A more precise analysis of the specificity of the suppression was carried out by using an HLA-B/D recombinant family (BAR) in a three-cell assay (Table 3). PLT II anti-DRw7 cells were added in combinations using all the members of the family BAR as stimulators. A specific suppression for the DRw7-bearing 'b' haplotype was observed within this family. The combination with the recombinant child C1 who has only inherited the HLA-D part of the b haplotype was also suppressed. The specificity of the suppression seems to be HLA-D region specific.

The apparent specificity of the suppression for the HLA-D-region products could involve either DR antigens or D products—if these are distinct determinants. However, in familial cases where an unusual association between HLA-D and DR specificities occurs on one HLA haplotype, the respective role of the two 'different' products could be studied separately. An example is shown in Table 4: in family LIS an unusual haplotype was recognised previously, in which DRw1 is associated with Dw4 (instead of the usually observed DRw1–Dw1 association). A PLT II twice primed against an haplotype DRw1 Dw1 was added in combinations where different stimulators were used, either DRw1 Dw1 or DRw1 Dw4 bearing cells. A specific suppression for the DRw1 antigen of the stimulator was observed to be independent of the associated Dw specificity.

Experiments with lymphocytes primed against two DR specificities were also carried out. These lymphocytes were

Table 2 Suppression follows the HLA haplotype

Responder	Stimulators irradiated		Third cells added irradiated		%
	HLA haplotype	HLA-DR	DEG C7 (c.p.m.)*	PLT II (c.p.m.)*	
DEG. F.	a/b	7/3 SC†	16,164	6,966	57
	M. c/d	5/–	4,795	4,231	12
	C1 a/c	7/5	20,820	5,394	74
	C2 a/c	7/5	17,996	5,461	69
	C3 a/c	7/5	13,295	5,117	61
DEG. C7 DRw 3/–	C4 a/d	7/–	13,181	3,934	70
	C5 b/c	3/5	3,844	6,684	–
	C6 b/c	3/5	3,060	3,286	–
	C7 b/d	3/– NC	740	1,412	–
	GIL.S	7/7	13,716	7,268	47
SCH.D	1/1		14,360	16,488	–

Culture conditions were as reported in Table 1. PLT II was produced as follows: responder, DEG C7 (DRw3/–), and stimulator irradiated father, DEG. F (DRw3/DRw7) were co-cultured for 10 d (PLT I). These cells were restimulated for 7 d with an irradiated homozygous cell (DRw7/DRw7) and gave PLT II cells. Three-cell assays were carried out with: (1) responder cell unprimed, DEG. C7 (DRw3/–) (5×10^4 cells); (2) irradiated stimulating cells with different DR specificities (5×10^4 cells); (3) third irradiated cells either PLT II or DEG (DRw3/–) (5×10^4 cells). The proliferative response was evaluated on day 7 of culture, and the per cent suppression calculated as in Table 1.

* Medium values of three cultures.

† SC, specific control; NC, negative control.

Table 3 Suppression follows the D part of a recombinant HLA-B/D haplotype

Responder	Stimulators irradiated			Third cells added irradiated		%
	HLA-A	B	DR	LER (c.p.m.)*	PLT II anti-DRw7 (c.p.m.)*	
BAR. F	a	28	14	–	34,442	15,192
	b	30	13	7		
	M. c	2	27	1	40,928	38,202
	d	3	35	5		
	C1 ab	28	14	7	28,107	20,789
LER. DRw 2/2	c	2	27	1		
	b	30	13	7	45,489	30,379
	C2 c	2	27	1		
	a	28	14	–	26,634	29,195
	b	2	27	1		
VAL. M	x	25	15	5	17,316	5,036
	y	1	44	7		
	z	3	7	2	1,412	1,817
LER. C	w	3	7	2		

PLT I produced with LER (DRw2/DRw2) as responder, and VDL M (DRw5/DRw7) as irradiated stimulator gave an anti DRw5 + DRw7 (PLT I) after 10 d. These cells sensitised a second time (7 d) against an homozygous irradiated cell (DRw7/DRw7) gave the PLT II cells. Three cell assays were carried out: (1) responder cell unprimed (LER DRw2/DRw2); (2) irradiated stimulating cells from members of family BAR. (in this family child C1 is a known HLA-B/D recombinant); (3) third irradiated cells, either PLT II (responder primed twice) or LER (DRw2/DRw2). The proliferative response was evaluated on day 7 of culture as for Table 2.

* Medium values of three cultures.

discriminative bispecific PLT reagents. Using repeated sensitisations with one or the other specificities, we sought to establish whether they could independently generate suppressors specific for one or the other DR antigen. For instance, DRw2/DRw2 lymphocytes were primed against cells possessing DRw5/DRw7 antigens for 10 days (bispecific PLT I) and co-cultured for 7 more days with either a DRw5/DRw5 or DRw7/DRw7 stimulator (PLTs II). The suppressive activity of these PLTs II was tested in a three-cell assay (Table 5). The bispecific PLTs restimulated by DRw5 antigens greatly suppressed the response against DRw5-positive cells, whereas the suppression of the response against DRw7-positive cells was lower. The opposite was observed when restimulation was carried out with DRw7 antigens. This suggests that there are two distinct classes of suppressors—one specific for DRw5-positive cells and the other for DRw7-positive cells.

Table 4 PLT II cells specifically suppress the response to DR antigens

				Third cell added irradiated			
Stimulators irradiated				PLT II			
Responder	HLA haplotypes	DR	D	DER. F (c.p.m.)*	anti-DRw1 (c.p.m.)*	% Suppression	
NC† 1	a/b	2/-	2/-	2,423	2,508		
	SC 2	a/c	2/1	2/1	43,594	8,024	81
	3	a/c	2/1	29,055	10,525	63	
	4	a/b	2/1	2/4	44,502	12,507	72
	5	b/c	1/5	4/5	38,018	12,911	66
DER F							
DRw w/-							
	6	1/1	1/1	30,355	10,170	66	
	7	7/7	7/7	42,496	30,237	29	
	8	5/7	5/7	30,853	28,144	9	
	9	2/7	2/7	31,572	24,581	22	
	10	5/-	5/-	31,674	37,555	—	

PLT I produced with DER. F (DRw2 Dw2/–) as responder and DER. C, (DRw2 Dw2/DRw1 Dw1) as irradiated stimulator, gave after 10 d an anti-DRw1 PLT I; sensitised a second time, with the same irradiated stimulator for 7 d it gave PLT II cells. Three-cell assays were carried out with: (1) responder cell unprimed; DER F (DRw2 Dw2/–); (2) irradiated stimulating cells from family members and unrelated donor's cells, (3) third irradiated cells, either PLT II (responder primed twice) or DER F, (DRw2 Dw2/–). HLA-D typing was carried out using HLA-D homozygous typing cells (7th Histocompatibility Workshop cells) in a standard micro MLR assay. The results were double normalised (according to the method of Ryder *et al.*¹⁸). DR determinants were as for Table 1.

* Medium values of three cultures.

† NC, negative control; SC, specific control.

Table 5 Suppressive activity of PLTs directed against two DR specificities and primed twice (PLT II), tested in a three-cell assay

Responder	Irradiated stimulating cells No.	DR	Control (LER 2/2) (c.p.m.)	PLT II (c.p.m.)	Third irradiated cell added		B % Suppression
					A % Suppression	PLT II (c.p.m.)	
LER	1	1/5	40,928	25,650	38	38,202	7
	2	2/5	15,700	8,878	44	17,840	—
	3	5/5	25,726	4,038	85	16,795	35
	4	3/5	30,006	9,287	70	15,947	52
	5	-/5	24,875	8,936	65	20,866	17
	6	5/5	38,093	9,212	76	27,355	29
	7	5/5	25,622	9,093	65	19,644	24
LER DRw2/DRw2	8	-/7	34,442	23,963	31	15,192	56
	9	1/7	28,107	34,439	—	20,789	27
	10	1/7	45,489	38,879	15	30,379	34
	11	3/7	16,125	22,330	25	18,014	32
	12	7/7	26,290	20,579	22	14,458	46
	13	-/7	22,983	25,749	—	15,363	34
	14	1/-	26,634	28,250	—	29,195	0
	15	-/3	30,350	26,801	12	27,682	9
	16	-/3	20,523	30,311	—	24,712	—
	17	3/3	20,807	27,683	—	26,702	—
	18	2/3	26,898	17,549	35	25,824	4
Negative control			2,770	1,654		2,940	

Bispecific PLT I was produced with LER DRw2/DRw2 as responder and VDL M (DRw5/DRw7) as irradiated stimulator. PLTs II were produced by sensitising PLT I cells a second time with either cells GIL (DRw7/DRw7) = PLT II A or cells MAI (DRw5/DRw5) = PLT II B for 7 d. The three-cell assay was carried out as follows with: (1) responder cell unprimed: LER (DRw2/DRw2); (2) γ -irradiated stimulating cells with different specificities (DRw5, DRw7, DRw3 . . .); (3) third γ -irradiated cell: either PLT II A or B (responder primed twice, see Table 2) or LER (DRw2/DRw2).

These experiments indicate that lymphocytes which have been sensitised twice *in vitro* contain active suppressor cells, capable of suppressing 30–80% of the allogeneic proliferative response of unprimed cells. These *in vitro*-generated suppressor cells are radioresistant and specific for the HLA-DR antigens or closely associated structures presented by the stimulator lymphocytes. The suppressive activity of lymphocytes primed against two HLA-DR antigens seems to be induced by two distinct populations of suppressor cells. These suppressors behave (at least for the specificity of the suppression) in the same way as described for lymphocytes from a multiparous woman^{8,9} which were spontaneous suppressors of the response against HLA-D-region products of her husband, and were also radioresistant. However spontaneous suppressors specifically restricted to HLA-A specificities have also been reported¹⁰.

In vitro-generated suppressor cells are probably T lymphocytes, as only T cells are known to proliferate in MLR; however, there is no direct evidence. Cell-mediated lymphocytotoxicity cannot explain the suppression phenomenon for at least two reasons. (1) In our system, cell division was inhibited by irradiation, which prevents reactivation of the cytotoxicity of the twice-primed lymphocyte population¹¹. (2) In contrast to the suppression observed here, the specificity of the lymphocytotoxicity has not so far been shown to be directed against HLA-DR antigens¹². An antigenic competition, even though unlikely, cannot be definitely excluded. We shall investigate a possible HLA restriction phenomenon between the responder and the suppressor lymphocytes as well as a possible suppression mediated by soluble factors.

These HLA-DR-specific *in vitro*-generated human suppressors are probably similar to the spontaneous suppressors described *in vivo* after pregnancy^{8,9}. On the other hand, this allogeneic phenomenon seems to be different from other suppressors reported in human, such as those induced by mitogenic stimulation^{13–16} and observed in various diseases¹⁷. The suppression described here can be considered as a balanced phenomenon involving interaction between different lymphocyte populations such as 'helper cells', responsible for the allogeneic proliferative response and 'suppressor cells', antagonists of helper cell activity. This phenomenon might be of great importance during pregnancy and in allograft tolerance.

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Postnatal cerebellar cells from staggerer mutant mice express embryonic cell surface characteristic

MORE than 100 mutations affecting neurological function have been described in mice¹. In one of these, staggerer^{2,3}, the development of normal synaptic relationships between cerebellar granule cells and Purkinje cells is disturbed^{4–6}. In a search for abnormalities in the cell surface of staggerer cerebellar cells,

we have measured cell agglutination in the presence of the plant lectins concanavalin A (Con A) and wheat germ agglutinin (WGA). We report here that cell surface properties necessary for WGA-induced agglutination are abnormal in the mutant cerebellar cell population whereas those necessary for Con A-induced agglutination are not. These results are consistent with the persistence of a characteristic of the embryonic cerebellar cell surface.

Wild-type and staggerer animals maintained on A C57-*+d se/sg ++* inbred background were obtained from the breeding colony of our department⁷. Homozygous staggerer offspring were identified by their black coat and neurological symptoms². All mice were decapitated on the seventh and tenth days after birth (P7, P10), P7 being the earliest age when staggerer animals could be identified. Wild-type littermates were identified by their dilute coat colour. Cerebella were removed and single cell suspensions were prepared by dissociation with trypsin-DNase followed by gentle trituration^{7,8}. Cell yield was $2-3 \times 10^6$ per P7 staggerer cerebellum and $1-2 \times 10^7$ per P7 wild-type cerebellum.

To allow recovery from brief exposure to trypsin during dissociation, cells were preincubated in Ham's F12 medium supplemented with horse serum (2%, Microbiological Associates) for 6 h at 35.5°C. The serum was removed by washing twice with phosphate-buffered saline (PBS, 600 r.p.m., 5 min, 4°C), and the cells were resuspended (1×10^6 cells per ml of PBS) for agglutination assays.

As described previously¹⁰ (summarised in Table 1), early embryonic wild-type cerebellar cells are highly agglutinable with both Con A and WGA. This agglutinability is lost, however, after about embryonic day 16, such that postnatal cerebellar cells require very high concentrations of the lectins for agglutination. In contrast, P7 and P10 staggerer cerebellar cells were highly agglutinable with WGA. Hapten-specific inhibition of staggerer cerebellar cell agglutinating activity (%) was measured in quadruplicate with WGA ($100 \mu\text{g ml}^{-1}$ in PBS) preincubated (15 min, 20°C) with the inhibitor. In those conditions, WGA-induced agglutination of P7 staggerer cerebellar cells was inhibited completely by *N*-acetyl-D-glucosamine (0.40 mM), *N*-*N*-di-*N*-acetylchitobiose (0.04 mM), chitin hydrolysate (prepared by Rupley's method¹¹ and approximately 0.02 mM, 1% w/v; hydrolysate demonstrated by paper chromatography to consist primarily of di- and trisaccharides) and ovomucoid (0.02 mg ml^{-1}). Agglutinating activity was not reduced by more than 50% by D-glucosamine (50.0 mM), D-galactose (50.0 mM), *N*-acetyl-D-galactosamine (50.0 mM) or methyl- α -D-mannoside (50.0 mM). These results agree with the reported carbohydrate binding specificity of WGA^{12,13}.

Table 1 Agglutination of staggerer and wild-type mouse cerebellar cells with Con A and WGA

Genotype	Age	WGA	Con A
Wild-type C57BL/6J	E13	50	200
	P0	450	800
	P3	>500	>1,000
	P7	>500	>1,000
	P10	>500	>1,000
Wildtype <i>+d se/+ d se</i>	P7	>500	>1,000
	P10	>500	>1,000
Staggerer <i>sg + +/sg + +</i>	P7	55	>1,000
	P10	48	>1,000

Values for C57BL/6J embryonic animals are taken from ref. 10. Values for postnatal wild-type and staggerer animals are given as lectin concentration ($\mu\text{g ml}^{-1}$) required for half maximal agglutination (75%). For details of the assay method see refs 9 and 10. Agglutination assays were carried out in quadruplicate. Results given are averaged values from four sets of assays, each set having been carried out with animals from a separate litter. 20 staggerer animals and 10 wild-type animals were studied. The variability among quadruplicate assays of lectin concentration required for half maximal agglutination was less than $15 \mu\text{g ml}^{-1}$.

Table 2 Recovery of P7 wild-type and P7 staggerer cerebellar cells from exposure to trypsin

Genotype		Con A ($\mu\text{g ml}^{-1}$)	WGA ($\mu\text{g ml}^{-1}$)
Wild-type <i>+d se/+ d se</i>	a)	150	50
	b)	>1,000	>500
	c)	160	65
Staggerer <i>sg + +/sg + +</i>	a)	150	50
	b)	>1,000	50
	c)	150	50

Lectin concentration ($\mu\text{g ml}^{-1}$) required for half maximal agglutination (75%). Agglutination assays were carried out in quadruplicate by the method of Burger⁹. a, Agglutination immediately following dissociation of cerebellar tissue into a single cell suspension^{7,8}. b, Agglutination of dissociated cells replaced in Ham's F12 medium supplemented with horse serum (2%, Microbiological Associates) for 6 h, at 35.5°C before the assay. c, Agglutination of dissociated cells replaced in growth medium as above for 6 h followed by exposure to trypsin (crystalline, Worthington, $10 \mu\text{g ml}^{-1}$ in PBS) for 10 min at 37°C. Subsequently ovomucoid ($10 \mu\text{g ml}^{-1}$ in PBS) was added, the trypsin was removed by washing the cells three times in PBS (600 r.p.m., 5 min, 4°C), and the cells were resuspended for agglutination assays. The variability among quadruplicate assays of lectin concentration required for half maximal agglutination was less than $15 \mu\text{g ml}^{-1}$.

As with early postnatal wild-type cerebellar cells, cells derived from staggerer mice were not agglutinable with Con A at P7 or P10 (Table 1). They could be agglutinated only by high lectin concentrations or by brief proteolysis as described below. The latter agglutination was specifically inhibited by methyl- α -D-mannoside (2.0 mM) but not by other haptens, in agreement with reports of Con A binding specificity¹²⁻¹⁴.

Interestingly, the cell surface abnormality in staggerer cerebellar cells was restricted to components involved in WGA-induced agglutination. In studies of wild-type cells from several brain regions, WGA-induced agglutination depended on the region of origin and age of the cells as well as the enzymatic treatment to which the cells had been subjected. Whereas cells from the embryonic cerebellum agglutinated with low concentrations of WGA¹¹, those from neural retina¹⁵, midbrain, medulla and cerebral cortex agglutinated only immediately after trypsinisation¹¹. The abnormality of the staggerer cerebellar cells could thus reflect the persistence postnatally of the early embryonic cell surface characteristics which result in high agglutinability by WGA.

This conclusion depends on the recovery by the cells from the effects of the trypsin used to prepare the cell suspension, and on the absence of large changes in the different cell populations caused by selective granule cell degeneration in mutant staggerer cerebellum. Evidence for the recovery of both wild-type and staggerer cerebellar cells from the effects of trypsin is presented in Table 2. Before replacing cells in growth medium for 6 h, P7 and P10 cerebellar cells collected from wild-type and staggerer animals were highly agglutinable with both Con A and WGA (Table 2a). This agglutination was hapten-specific, Con A-induced agglutination being inhibited exclusively by methyl- α -D-mannoside^{12,16} (2.0 mM) and WGA-induced agglutination by *N*-acetyl-D-glucosamine. After incubation in growth medium for 6 h, the Con A- and WGA-induced agglutinability of wild-type cells and the Con A-induced agglutinability of staggerer cells decreased markedly (Table 2b). To directly assess the response of recovered cells to trypsin, we subjected them to a second mild proteolysis. The result was a second increase in the Con A- and WGA-induced agglutinability of wild-type cells and in the Con A-induced agglutinability of staggerer cerebellar cells. A second exposure to trypsin did not further increase the WGA-induced agglutination of staggerer cells (Table 2c).

These results are consistent with the effects of brief exposure to trypsin on the response of several cell types to Con A and WGA. Trypsin reversed non-agglutinability but did not affect

high agglutinability¹⁶⁻¹⁹. Trypsin also has a transient effect on 3T3 cells, BHK cells and chick embryo fibroblasts, which become refractory to increased lectin-induced agglutinability within 6 h of the removal of the trypsin²⁰⁻²².

At P7, the age with which we are chiefly concerned here, the granule cell population of staggerer is 50% smaller than that of wild-type cerebellum. The other major cell populations, Purkinje cells and glial cells, are not different for staggerer^{24,25}. The three- to fivefold decrease in total cell yield we obtained for staggerer cerebellum was therefore due primarily to the selective loss of granule cells. To assess whether a smaller proportion of granule cells in the cell suspension influenced our agglutination results, we resuspended wild-type cerebellar cells at 3×10^6 instead of 1×10^6 cells per ml for agglutination assays. Agglutination of this cell suspension by Con A and WGA was identical to that reported in Table 1. It is therefore unlikely that dilution of other cell populations by the larger granule cell population in wild-type cerebellum exclusively explains the difference in WGA-induced agglutination found for staggerer cerebellar cells. This interpretation is strengthened by recent studies showing that lectin-induced agglutination can be used to separate out a subpopulation of undifferentiated cells from a mixed population of cloned embryonal carcinoma cells²⁶.

Because the agglutination assays were carried out in suspension, it is not possible to identify the cell type in the staggerer cell population which is the site of the abnormality in WGA-induced agglutination. We shall investigate this by electron microscopy of agglutinated cells and by histochemical studies of WGA-binding to frozen tissue sections.

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Erythroid bursts produced by Friend leukaemia virus *in vitro*

WHEN mouse haematopoietic cells are incubated in plasma clot or methyl cellulose cultures in the presence of erythropoietin (EP), at least two kinds of colonies are observed¹⁻⁴. After 2 days, randomly distributed erythroid colonies (defined as a clone of eight or more benzidine-positive cells) appear^{1,2}, and after 3-8 d of culture with higher concentrations of EP, larger colonies arise in clusters or 'bursts'^{3,4}. The cells of origin have been termed the colony-forming unit-erythroid (CFU-E)¹ and burst-forming unit-erythroid (BFU-E)^{3,4}, respectively. Erythropoiesis in mice is also increased considerably by the Friend virus complex^{5,6}, which has been studied mostly *in vivo* although there are systems for the study of erythroid transformation *in vitro* soon after infection⁷⁻¹⁰. Nooter and Bentvelzen¹¹, using methyl

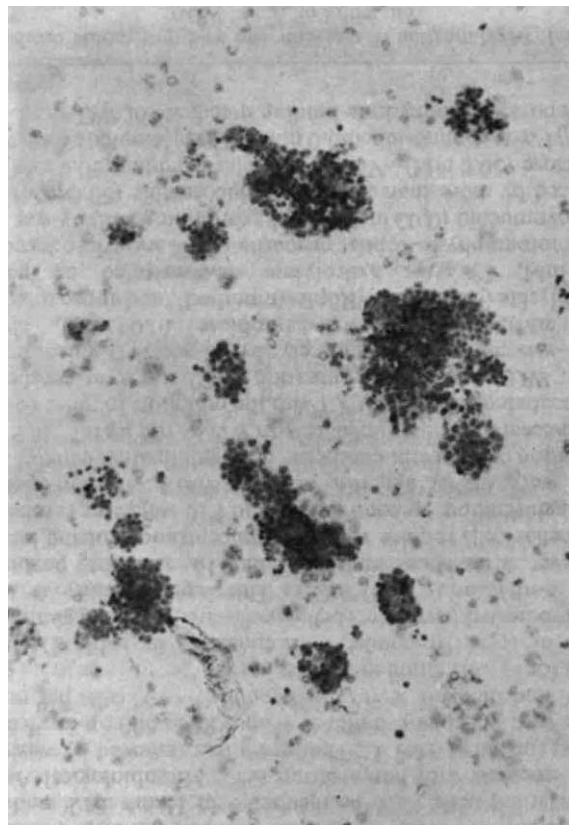


Fig. 1 Erythroid bursts produced by Friend virus infectious plasma. BALB/c mice were given three intraperitoneal injections (0.04 mg per g) of phenylhydrazine hydrochloride 62, 48 and 40 h before killing. When the mice had been killed, marrow cells were flushed from the tibias and femurs with sterile Hanks balanced salt solution (HBSS). The cells were washed twice with HBSS and collected each time by centrifugation. 4×10^6 nucleated cells were resuspended in 0.3 ml of a 1:20 dilution of Friend virus infectious plasma in alpha medium (7,500 spleen focus-forming units¹⁸ and 1.5×10^5 XC plaque forming units¹⁹). This cell suspension was incubated at 4 °C for 1 h. The virus-cell mixture was then diluted to 2 ml to give the following final concentrations: bovine serum albumin, 1% v/v; penicillin, 100 IU ml⁻¹; streptomycin, 100 µg ml⁻¹; β-mercaptoethanol, 10⁻⁴M; fetal calf serum, 30% v/v; beef embryo extract, 0.85% v/v; bovine citrated plasma, 10% v/v. This mixture was pipetted into triplicate 0.5 ml tissue culture wells (Disposo-Trays, Model 96-SC, Linbro) and incubated at 37 °C in humidified atmosphere of 5% CO₂-95% air. After 5 d of incubation, the clots in the wells were placed on glass slides, fixed with 5% glutaraldehyde and stained with 3,3'-dimethoxybenzidine and Harris haematoxylin³.

cellulose cultures, showed that mouse marrow cells, previously incubated *in vitro* with purified Rauscher leukaemia virus, develop more erythroid colonies than untreated control cells. Clarke *et al.*¹², using the plasma clot method, observed erythroid colonies 2 d after incubation of mouse marrow cells with culture fluid from Friend virus infected monolayers of fibroblasts. These colonies were similar to EP-induced 2-d colonies in gross morphology and random distribution throughout the clot. Virus-induced bursts were not present in later stages of culture and erythroid colonies were not induced after incubation of marrow cells with plasma from Friend virus infected mice. We now report that when mouse marrow cells are incubated *in vitro* with infectious plasma or tissue culture fluids containing the Friend virus complex, a substantial number of erythroid bursts appear 5 d after the initiation of the cultures. The Friend virus complex consists of at least two biologically active viruses—the spleen focus-forming virus (SFFV) which is replication defective and essential for increased erythropoiesis *in vivo*, and a helper murine leukaemia virus (MuLV-F)^{13,14}.

The time elapsed between primary infection with Friend virus complex *in vivo* and the peak of haemoglobin synthesis *in vitro* is 4–5 d (ref. 15). The size of the peak depends on the dose of virus and the time after infection at which the cells are placed *in vitro*, but the time course of haemoglobin synthesis is constant. Although these results were obtained in liquid cultures and with ⁵⁹Fe which was incorporated into haem¹⁵, Friend virus-infected marrow cells might be expected to produce benzidine-positive cells in semi-solid cultures within a period similar to that required for the wave of ⁵⁹Fe incorporation.

Marrow cells from mice treated with phenylhydrazine to make them anaemic, were incubated with a 1:20 dilution of Friend virus plasma and seeded in plasma clot cultures. A typical 5-d burst is shown in Fig. 1. We observed similar but fewer bursts with marrow cells from normal mice. In contrast to erythroid colonies that appear 2 d after the addition of EP, almost all colonies seen 5 d after the addition of Friend virus complex were in clusters resembling the bursts produced by EP^{3,4}. Each cluster consisted of multiple colonies of erythroblasts containing from 8 to more than 100 cells. It has been shown that all the colonies of an EP-induced burst arise from a single cell¹⁶. Whether the Friend virus complex acts on the same cell that responds to EP with the formation of 5-d bursts remains to be determined.

As shown in Table 1, the number of 5-d bursts decreased progressively as the cells were incubated with increasing dilutions of infectious plasma. A constant association was observed between burst forming ability and focus-forming ability in two separate infectious plasmas and cell culture supernatants from 3 different cell lines producing SFFV. In contrast, burst forming activity was not detected in normal BALB/c mouse plasma or cell culture fluids from cell lines which produced only MuLV.

Two sets of results (Table 1 and Fig. 2) suggest that the development of erythroid bursts after the addition of Friend virus infectious plasma is a direct effect of virus on the marrow cells. First, heat treatment at 56 °C for 1 h rendered Friend virus infectious plasma incapable of inducing splenic foci *in vivo* and producing splenomegaly^{5,6,15}. Heat treatment of the infectious plasma either at 80 °C for 3 min or at 56 °C for 1 h abolished the formation of 5-d bursts *in vitro* (Table 1). EP is heat stable¹⁷ and similar treatment did not destroy its ability to induce erythroid colonies from mouse marrow cells after 2 d of culture in the plasma clot system (our unpublished results). Second, Friend virus infectious plasma was layered over a 5–20% linear sucrose gradient, and an EP preparation was layered on an identical gradient (Fig. 2). Both gradients were centrifuged and fractionated. Each fraction of the gradient containing infectious plasma was assayed for its ability to induce 5-d erythroid bursts, and for its content of SFFV and MuLV-F as defined by the spleen focus-forming assay¹⁸ and XC assay¹⁹, respectively. Most of the 5-d erythroid burst-forming activity, as well as XC plaque and spleen focus-forming activities, were recovered in the bottom half of the gradient. There was no activity at the top,

Table 1 Relation of SFFV activity to production of 5-d erythroid bursts

Test preparation	Dilution	Contains SFFV	Contains MuLV-F	Average 5-d bursts per clot
Expt 1				
M19 plasma	1:20	+	+	23
M19 plasma treated, 56 °C × 1 min.	1:20	—	ND*	0
M18 plasma	1:20	+	+	30
M18 plasma	1:40	+	+	20
M18 plasma	1:80	+	+	12
M18 plasma	1:160	+	+	9
M18 plasma	1:320	+	+	2
M18 plasma	1:640	+	+	1
M18 plasma treated, 80 °C × 3 min	1:20	—	ND	0
Normal BALB/c mouse plasma	1:20	—	ND	0
Expt 2				
Tissue culture supernatants:				
SC-1† (M16)	Undiluted	+	+	11
FP52‡	Undiluted	+	+	23
EY26§	Undiluted	+	+	3.6
SC-1 (M16) treated, 56 °C × 60 min	Undiluted	—	ND	0
FP52 treated, 56 °C × 60 min	Undiluted	—	ND	0
EY26 treated, 56 °C × 60 min	Undiluted	—	ND	0
SC-1† MuLV-M	Undiluted	—	+	0
SC-1† MuLV-F	Undiluted	—	+	0
1902§ Nonproducer cells	Undiluted	—	—	0

In expt 1, marrow cell suspensions were prepared from mice pretreated with phenylhydrazine as in Fig. 1. The cell suspensions were mixed with the indicated test preparations and incubated as described in Fig. 1. M19, M18 and M16 refer to separate pooled plasmas prepared from Friend virus-infected mice as previously described¹⁵. In expt 2, marrow was prepared from normal BALB/c mice. EY26 cells are '1902' cells (nonproducer cells that contain SFFV genetic information²², but do not release virus particles) which were superinfected with cloned murine leukaemia virus to rescue SFFV²². FP52 cells were developed by infection of mouse fibroblasts with Friend virus²¹. SC-1 cells²⁰ were infected with Moloney leukaemia virus obtained from the ATCC MD {SC-1 (MuLV-M)}; M16 Friend virus infectious plasma prepared as previously described¹⁵ {SC-1 (M16)}; or M16 plasma at a dilution that is past the end point of SFFV {SC-1 (MuLV-F)}.

* ND, not determined

† SC-1 cells²⁰ infected with an infectious plasma preparation (M16), MuLV-Moloney or MuLV-Friend.

‡ A gift from Dr Alan Bernstein².

§ A gift from Dr David Troxler²².

where EP would have been located. Thus, erythroid burst formation was produced by an entity that sedimented with the known virus activities in Friend virus preparations. To detect EP activity, fractions of the EP gradient were tested for their ability to induce 2-d erythroid colonies. As expected, many colonies were produced by fractions in the top part of the EP gradient, but only small, background, numbers were produced by lower fractions.

Thus, we have shown that when Friend virus complex is incubated *in vitro* with mouse marrow cells erythroid bursts develop 5 d later. Bursts can also be seen when the clots are removed on days 3–8, and the timing for the optimum number of bursts remains to be determined. The production of erythroid bursts after incubation of haematopoietic cells with infectious plasma provides an *in vitro* method for studying the interaction of the Friend leukemia virus(es) with the target cell(s). Various indirect *in vivo* approaches have been used to define the cells which are the targets for erythroid transformation by the Friend and Rauscher viruses. A direct *in vitro* approach is now possible,

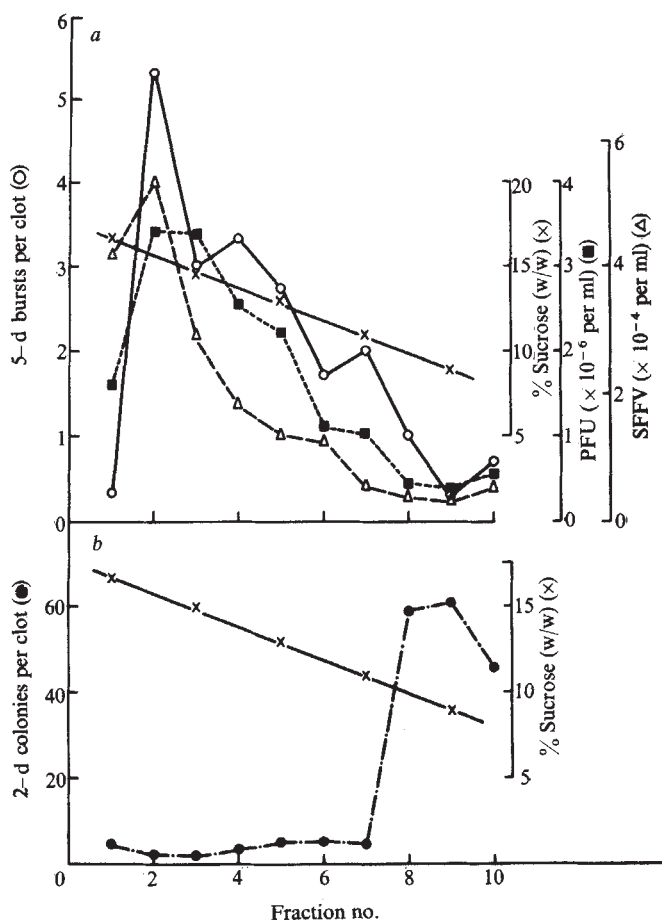


Fig. 2 Recovery of 5-d erythroid burst-forming activity after velocity gradient sedimentation of Friend virus infectious plasma. One ml of infectious plasma was layered over a 4.5 ml 5–20% w/v linear sucrose gradient and centrifuged at 45,000 r.p.m. for 7 min at 4°C in an SW50.1 rotor. An erythropoietin preparation (EP, Step III, Connaught Medical Research Laboratories) was centrifuged on a similar gradient. Fractions of 0.5 ml were collected from the bottom of both gradients. Marrow cells from phenylhydrazine-treated mice were incubated as in Fig. 1 with each fraction which was diluted 1:10 with alpha medium and filtered through 0.45-µm filters. Plasma clots were examined for colonies or bursts at 5 d (a) and at 2 d (b). In (a) the fractions were tested in the spleen focus-forming¹⁸ and XC plaque-forming assays¹⁹.

and experiments are in progress to delineate these cells. This is the first demonstration of the production of erythroid bursts *in vitro* by the Friend virus complex. Because the effect of the virus in our system is quite large (each cluster consists of multiple large colonies) we have now a better means of studying the process of erythroid transformation by a murine virus. It should also be possible to develop a suitable *in vitro* assay for SFFV.

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Mutagenesis of Chinese hamster cells is facilitated by thymidine and deoxycytidine

ONE-WAY information transfer from nucleic acids to proteins is generally considered to ensure accurate cell self-duplication, in which errors occur by mutation¹. However, it has been argued that self-duplication is a multimolecular process consisting of several potentially error-prone stages², and that mutation is a complex cellular process³. Moreover, it has been shown that error-prone DNA polymerases cause mutations in bacteriophage T₄ (ref. 4,5). We now report that mutations produced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), at two separate loci in Chinese hamster cells, are increased 3–10-fold when the cells are treated in the presence of low concentrations (~2 × 10⁻⁵ M) of thymidine (TdR) and deoxycytidine (CdR). We conclude that imbalanced nucleotide pools facilitate errors of replication of methylated DNA, and suggest that aberrant synthesis of macromolecules, facilitated by imbalances in precursor pools, plays an important part in the expression of genetic damage.

Figure 1a shows that in the presence of CdR or TdR (0.5–2.0 µg ml⁻¹) the frequency of ouabain-resistant (Oua^r) colonies induced by MNNG was increased 2–15-fold, the most consistent increase (2.9–3.0-fold) occurring in the presence of equal concentrations of TdR and CdR. Figure 1b shows that in the presence of CdR and TdR the frequency of 8-azaguanine-resistant (AzG^r) colonies induced by MNNG was consistently increased 2.7–4.3-fold. However, these nucleosides did not increase the yield of AzG^r or Oua^r colonies that arose spontaneously in the two experiments shown in Fig. 1. The spontaneous frequencies were ~1 × 10⁻⁵ survivors for AzG^r and Oua^r colonies.

Figure 1c shows that cytotoxicity of the LD₅₀ level of MNNG (1.0 µg ml⁻¹) was greatly increased when cells were grown and treated in dialysed fetal calf serum in the presence of as little as 0.5–2.0 µg ml⁻¹ of TdR. This effect did not occur in medium containing the same concentrations of both TdR and CdR. In medium containing CdR in the absence of TdR, MNNG was ~20% less toxic than in medium to which no nucleoside had been added. In the absence of MNNG, CdR and TdR, separately, at concentrations of 0.5–2.0 µg ml⁻¹ reduced the plating efficiency by only 7–17%.

The results shown in Fig. 1a and b are surprising, because cells possess controls to ensure that the DNA replication mechanism selects the correct nucleotides⁶. Therefore, it is necessary to inquire whether the colonies scored for Fig. 1a and

b represent increased mutation frequencies, selective toxicity of MNNG for non-mutant cells, selective growth of mutants, decreased time for expression of the mutant phenotypes, or modification of MNNG or its cellular targets in the presence of CdR and TdR. Experiments designed to test these possibilities have been carried out and are summarised below. They will be reported in detail elsewhere.

AzG^r clones have been extensively characterised in previous studies in our laboratory^{7,8}. Of 20 AzG^r colonies from V79 cells treated with MNNG in the presence of TdR and CdR (4.0 µg ml⁻¹), 70% showed the AzG^r, HAT (10⁻⁵ M hypoxanthine, 10⁻⁶ M aminopterin, 5 × 10⁻⁴ M TdR) sensitive (HAT^r) phenotype characteristic of hypoxanthine: guanine phosphoribosyl transferase (HGPRT) deficient mutants^{7,8}, 5% showed the AzG^r, HAT^r phenotype found in mutants containing HGPRT with an altered *K_m* for substrate^{9,10}, and 25% were unable to grow to confluence in either AzG or HAT. None of the isolates showed the AzG^s, HAT^r phenotype of the parental non-mutant cells. Therefore, we consider all the colonies that were scored for Fig. 1*b* to be mutants. Of 17 Ou^r colonies isolated, all remained completely resistant to 3 mM ouabain 8 and 35 d after isolation. Therefore, we consider the Ou^r phenotype to be characteristic of mutants containing a modified (Na⁺, K⁺)ATPase^{11,12}.

Both in the presence of CdR, or CdR plus TdR, and in their absence, cells treated with 0.5 µg ml⁻¹ of MNNG underwent the same number of doublings (3–3.5) before exposure to ouabain or AzG. Moreover, we have measured the AzG^r colony frequency over 12 population doublings in cultures treated with the LD₅₀ level of MNNG in the presence or absence of CdR, TdR and CdR plus TdR (2.0 µg ml⁻¹). In those experiments, the mutation frequencies in cultures grown in the presence of CdR and TdR remained three- to seven-fold higher than the frequencies obtained with MNNG alone. These findings show that the increased mutation frequencies produced by CdR and TdR are not the result of differences in expression time.

The doubling time (12.7 ± 0.66 h) of AzG^r colonies isolated from cultures treated with MNNG in the presence of CdR plus TdR was not different from the doubling time (12.4 ± 1.5 h) of AzG^s cells in medium containing CdR plus TdR (2.0 µg ml⁻¹). Therefore, the AzG^r mutants did not have a selective advantage over AzG^s cells in medium containing CdR and TdR.

In the absence of TdR, concentrations of CdR ranging over 0.5–2 µg ml⁻¹ produced the same slight (<20%) reduction in MNNG cytotoxicity (Fig. 1*c*), showing that the CdR was not preventing the MNNG from reaching targets for cytotoxicity within the cells.

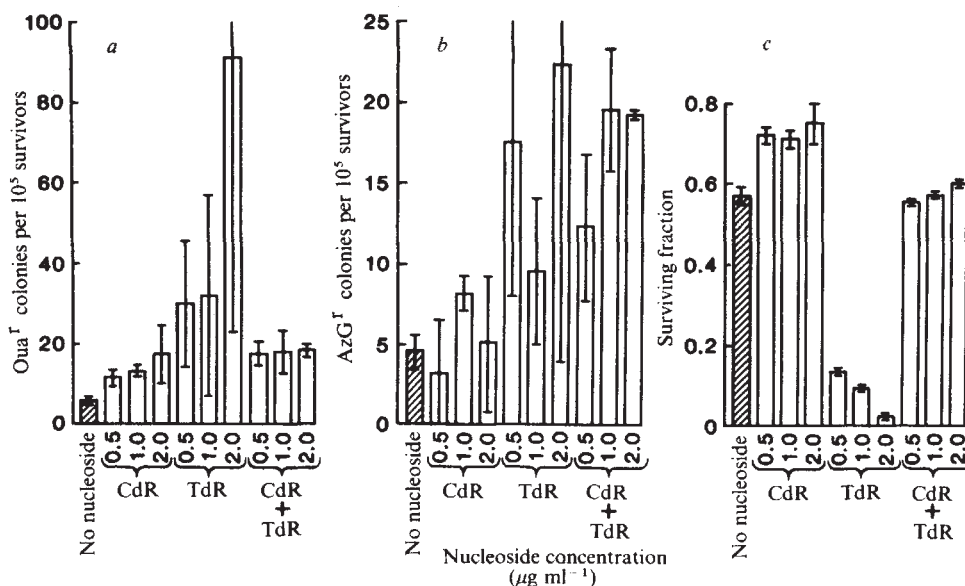
Moreover, DNA single-strand breaks produced by MNNG were measured using our alkaline sucrose-gradient procedure^{13,14}; the extent and also the persistence of the breaks produced in the presence of the CdR plus TdR (2.0 µg ml⁻¹ of each) or in the absence of the nucleosides were not significantly different. Therefore, neither CdR nor TdR prevented MNNG from reaching the DNA. Thus, we consider it most unlikely that the effects of the nucleosides on cytotoxicity and mutation result from modification of MNNG or its cellular targets before alkylation occurs. We must therefore conclude that MNNG-induced mutagenesis at two different loci (one, recessive, that involves purine nucleotide metabolism, and the other, dominant, that involves cation transport through the cell membrane) in Chinese hamster V79 cells, was increased three- to four-fold in the presence of 0.5–2.0 µg ml⁻¹ of CdR plus TdR. Moreover, we have obtained similar results using *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (ENNG) and ethyl methane sulphonate (EMS) as mutagens (A.R.P., H.P. and C.H., unpublished data).

The MNNG-induced mutation frequency increases with increase in the toxicity of MNNG in Chinese hamster cells^{7,8,15,16}, so the increased mutation frequency observed in the presence of TdR could have been associated with the greatly increased toxicity with TdR (Fig. 1*c*). However, when both CdR and TdR were present, the TdR sensitisation of the cells to the cytotoxicity of MNNG was abolished (Fig. 1*c*), although the

Fig. 1 The induction by MNNG of Ou^r (*a*) and AzG^r colonies (*b*), and the cytotoxicity of MNNG (*c*), in the presence of CdR and TdR in cultures of V79 Chinese hamster cells. For one assay of the induction of AzG^r and Ou^r colonies, 10⁵ cells were plated in each of two 100-mm plastic dishes (Corning) in medium of the same composition as used for the cytotoxicity assays. After incubation for 6 h, the cells were treated with 1.0 µg ml⁻¹ of MNNG for 2 h, incubated for 48 h in MNNG-free medium containing the indicated supplements of CdR and TdR, trypsinised, replated in fresh medium of the same composition at a density of 0.5–1 × 10⁵ cells per plate in medium containing appropriate supplements, incubated for 24 h, and treated with AzG (40 µg ml⁻¹, 0.26 mM) or with ouabain (3 mM). The cultures were incubated for 10–12 d with a change of fresh AzG- or ouabain-containing medium supplemented with the indicated levels of CdR and TdR at 5 d. Colonies were fixed, stained and scored 8–10 d later using the same procedures as those used in the cytotoxicity assay. For one assay of cytotoxicity, 200 cells were plated in each of four 55-mm plastic dishes (Corning) in Dulbecco's medium supplemented with 10% dialysed fetal calf serum (DS) (Gibco), or in DS plus various concentrations of TdR and CdR. The dishes were incubated for 6 h at 37 °C in a humidified atmosphere of 10% CO₂ in air. Some dishes received 1.0 µg ml⁻¹ of MNNG from a stock solution in acetone, others, controls, received 0.25% acetone. After a 2-h incubation, the medium was aspirated and replaced with fresh medium free of MNNG, but containing the supplements of CdR and TdR. The cultures were then incubated for 7–9 d, fixed, stained with Giemsa, and the colonies were counted. Only colonies with diameters of 0.5 mm or more were scored using a Biotran automatic colony counter (New Brunswick Scientific). Mutation frequencies (*F*) were calculated by the formula:

$$F = \text{total mutant colonies} / (\text{total cells replated} \times \text{plating efficiency of replated cells}),$$

and also by applying Poisson statistics as described in ref. 29. Both methods of calculation gave essentially the same results. The results shown here are the means and ranges of two assays.



yield of Ouaf and AzGf colonies was reproducibly three- to four-fold greater than with MNNG alone (Fig. 1a, b).

It is well established that exogenous TdR and CdR, supplied to cell cultures in concentrations of the order of 10^{-5} M ($\sim 2.0 \mu\text{g ml}^{-1}$) produce an imbalance of the deoxyribonucleotide pools in many lines of mammalian cells, including Chinese hamster ovary cells^{17,18}. Moreover, recent studies in cell-free systems show that the fidelity of DNA replication decreases as the relative concentration of incorrect nucleotides increases in the reaction mixture¹⁹. Therefore, we offer the following interpretation of our data: in V79 cells the imbalance produced in nucleotide pools by the uptake of exogenous TdR and CdR facilitates aberrant replication of DNA alkylated by MNNG, ENNG and EMS. Some errors are lethal, others are mutagenic. The lethal errors are facilitated by TdR, but not by CdR; the mutagenic errors are facilitated by both TdR and CdR.

Our interpretation seems at first to be incompatible with the fact that living systems possess vital 'proof-reading' mechanisms which ensure high fidelity of DNA replication⁶. However, it can be argued that mechanisms which promote infidelity in response to life-threatening environments will be advantageous to living organisms. Radman²⁰ has proposed such an 'S.O.S.' mechanism; it is considered to operate by mutagenic DNA repair induced in response to genetic damage.

Evidence for a mechanism that need not involve DNA repair, for producing infidelity in DNA replication, is provided by our data (Fig. 1), and by experiments showing increased mutation in Chinese hamster cells grown in medium containing high (4×10^{-3} M) concentrations of thymidine²¹, and thymine-starved bacteria^{22,23} and yeast²⁴, which show that DNA synthesis is increasingly error prone when the pools of DNA precursors become unbalanced.

DNA is not the only macromolecule whose synthesis can be made error prone by unbalancing its precursor pools. Starvation of amino acids leads to severe translational errors in bacterial and animal cells²⁵. Bujard and C.H.²⁶ found that *in vitro* transcription of synthetic DNA templates by *Escherichia coli* RNA polymerase became error prone in the presence of 4×10^{-8} M uridine triphosphate, which by nearest-neighbour analysis was shown to be incorporated into RNA in place of cytidine triphosphate. Thus, not only the synthesis of DNA, but also those of RNA and protein can be error-prone in the presence of unbalanced precursor pools, and we propose that such errors could become manifested as substantial alterations in gene expression. The accumulation of copying errors in macromolecule synthesis has been suggested as a mechanism for ageing²⁷, and has been implicated in mutagenesis and carcinogenesis^{19,28}. We consider it likely that errors in DNA, RNA and protein synthesis, facilitated by imbalances in precursor pools, are not only involved in carcinogenesis, mutagenesis and ageing, but have also played a part in the evolution of species.

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Transformed cell lines susceptible or resistant to *in vivo* surveillance against tumorigenesis

THE transformation of a normal cell to one which grows as a cancer requires several steps, one of which may involve the escape from tumour surveillance¹. We describe here an experiment designed to define these steps more clearly. The principle is to transform normal continuous cell lines *in vitro* and then select for transformants by their growth in agarose^{2,3}. These procedures avoid any host selection. By testing the tumorigenicity of these cloned, transformed cell lines in normal and immunodeficient mice, it would be possible to estimate the frequency of transformation resulting in tumorigenic clones and the proportion of these clones that is rejected by normal mice. Based on their tumorigenic potential, we have divided these cells into three classes (Table 1), 0, I, and C. 0 cells, although able to grow in agarose, do not form tumours in either test animal. I cells form tumours only in immunodeficient mice and C cells form tumours in both types of mice. The existence of 0 and I cells indicates that at least one additional mutation is required for these cells to grow as a cancer (C).

Table 1 Classification of cells by growth in agarose and mice

Type	Colony formation in agarose	Growth as tumour in Immune deprived mice	Normal mice
N	—	—	—
0	+	—	—
I	+	+	—
C	+	+	+

N cells were grown in Dulbecco's modified Eagle's minimum essential media (DME) plus 10% fetal bovine serum (FBS). These cells were treated with either virus (SV-40 or Abelson murine leukaemia virus) or chemicals (20-methylcholanthrene; *N*-methyl-*N*-nitro-*N*-nitrosoguanidine; 11,12-dihydro-3-methylcholanthrene-11,12-epoxide; or 5,6-dihydrobenz(a)anthracene-5,6-epoxide). 24 h later these cells were plated in 0.3% agarose containing DME plus 25% FBS at 10^5 , 10^4 and 10^3 cells per 60-mm plate. The plates were incubated at 37 °C until macroscopic colonies appeared, but no longer than 8–12 weeks. Colonies were picked from the agar and grown in DME plus 10% FBS. 5 or 10×10^6 cells were injected subcutaneously into normal mice and mice which had been thymectomised as adults, lethally irradiated (750 rads) and restored with 10^7 syngeneic fetal liver cells (AT × FL).

Table 2 Distribution of transformed clones characterised by inoculation into normal and AT $\times$ FL mice

Transforming agent	Classification of transformed clones			Efficiency of rejection*
	0	I	C	
Viral	14% (2/14)	64% (9/14)	21% (3/14)	75% (9/12)
Chemical	10% (2/21)	19% (4/21)	71% (15/21)	21% (4/19)

The distribution of chemically transformed clones into the 0, I or C categories differs significantly ($P < 0.005$)⁷ from the distribution of the virally transformed clones.

* Efficiency of rejection = no. of I clones/no. of I + C clones.

Cloned, continuous lines of normal fibroblast cells (N cells) were derived from BALB/c, BALB.B and C57BL/6 embryo cultures. These cells do not form colonies in agarose or grow as tumours even in immunodeficient mice (thymectomy, lethal irradiation and fetal liver restoration of adults; AT $\times$ FL). N cells were transformed with either viral or chemical carcinogens (Table 1) and colony formation in agarose was used to select for transformed variants.

As shown in Table 2, 35 independently derived transformed clones were tested for their tumorigenicity in syngeneic normal and immunodeficient mice. Of the 35 cell lines, 14 were derived following viral transformation and 21 were obtained after chemical carcinogenesis. Overall, 31 of 35 clones isolated from agarose formed tumours in AT $\times$ FL mice (that is, I + C lines), indicating a high correlation (89%) between *in vitro* growth in agarose and *in vivo* tumorigenicity. The 10–14% of transformed clones which were classified as 0 may represent a class of cells which are either inherently nontumorigenic or which are susceptible to surveillance mechanisms remaining in AT $\times$ FL mice. The collection of 0 lines is now being tested for tumorigenicity in mice which have been rendered deficient in other possible surveillance mechanisms.

From the data shown in Table 2, we can calculate that the proportion of tumorigenic transformed cell lines falling into the I-cell category was 9/12 (75%) for the virally transformed lines and 4/19 (21%) for the chemically transformed lines. Expressed in terms of the efficiency of surveillance, 75% of the viral and 21% of the chemically transformed cell lines were susceptible to a surveillance mechanism which is impaired in the AT $\times$ FL mouse. Although the AT $\times$ FL mouse is severely depleted of T-cell activity, and therefore, immunodeficient^{4,6}, these animals also have lower levels of natural killer (NK) cell activity. It is tempting to speculate that these deficiencies allow the growth of I lines.

We have not confirmed that all C lines are derived from I-type intermediates as predicted in the N $\rightarrow$ I $\rightarrow$ C sequence required by surveillance models. However, it is clear that clones which have undergone the mutation N $\rightarrow$ I require another mutation, I $\rightarrow$ C, to grow as a tumour in normal mice. We have several examples where this has occurred.

There are three potential sources of error in categorising I lines. First, the 0 lines could, in fact, be I lines if the AT $\times$ FL mouse had residual tumour resistance. Second, if N lines had acquired rejection antigens before transformation, these antigens could result in overestimation of the frequency of I lines; however, the high frequency of C lines makes this unlikely. Third, the transformed clones were tested at 10⁷ cells per animal for classification of tumour potential. If surveillance against tumours is of any physiological importance, the system must be capable of eliminating tumour cells when they are in relatively low numbers (certainly less than the test dose of 10⁷). At the test dose, efficiency of rejection could have only been underestimated.

To our knowledge, this is the only experiment in which the frequency of appearance of the postulated N $\rightarrow$ I step has been evaluated by the isolation of transformed clones in the absence of an immune system (that is, *in vitro*) and by testing them simultaneously in immunodeficient (AT $\times$ FL) and normal mice.

The existence of I cells shows that a host protective mechanism is operative. Although the difference between viral and chemical carcinogenesis seems large, the above sources of error would lead to an underestimation of the efficiency of this host protective mechanism. The resolution of these limitations may only decrease the difference. Consequently, we conclude that immune protective mechanisms have a significant but not absolute role in tumorigenesis.

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Influence of cyclic AMP on intracellular pH regulation and chloride fluxes in barnacle muscle fibres

THE pH sensitivity of numerous intracellular processes requires the close regulation of internal pH (pH_i). As the pH_i of many cells is maintained at a higher value than it would have if H^+ were at equilibrium across the cell membrane, it follows that the acidifying effects of cellular metabolism and of passive H^+ influx and HCO_3^- efflux must be balanced by active acid extrusion. It is now generally accepted that this extrusion is stimulated by decreases in pH_i , and that, in several cells, it is accomplished by exchanging external HCO_3^- for internal Cl^- (refs 1–4). Recently, a role for cyclic 3',5'-adenosine monophosphate (cyclic AMP) in pH_i regulation was suggested by the observation that dibutyryl cyclic AMP attenuates the CO_2 -induced fall of pH_i in mammalian cardiac muscle⁵. We now report experiments on the effects of cyclic AMP on acid extrusion and on accompanying Cl^- fluxes in the barnacle muscle fibre, a preparation known to contain cyclic AMP (ref. 6). Our data show that internally applied cyclic AMP enhances the response of the pH_i -regulating system to acid loads.

The experiments were carried out on superfused single muscle fibres of the giant barnacle (*Balanus nubilus*). Intracellular pH and concentrations of low molecular weight solutes were controlled by insertion of a porous dialysis capillary through the fibre, and perfusion with fluid⁷. In one series of experiments, pH_i was monitored by placing pH-sensitive and reference micro-electrodes into the fibre alongside the dialysis tube. In another series, either Cl^- influx or efflux was measured with ³⁶Cl.

The influence of cyclic AMP on the rate of acid extrusion was examined as shown in Fig. 1A. The fibre was loaded with acid by

dialysing with a cyclic AMP-free solution at pH 6.0 (Fig. 1A, segment *ab*); to reduce acid extrusion rate while acid loading, external pH (pH_o) was simultaneously lowered from 7.8 to 6.8 (ref. 8). When pH_i had fallen to 7.02 (Fig. 1A, point *b*), dialysis was halted so that acid no longer entered the cell by this route, and pH_o was returned to 7.8. In the absence of cyclic AMP, pH_i recovered at a low rate (Fig. 1A, segment *bc*). However, when the fibre was dialysed with an acid solution containing cyclic AMP (Fig. 1A, *cd*), pH_i recovered nearly four times faster (Fig. 1A, *de*). This recovery was blocked (Fig. 1A, *fg*) by SITS (4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonic acid), a potent inhibitor of acid extrusion^{1,4,9}. Figure 1B shows the effect

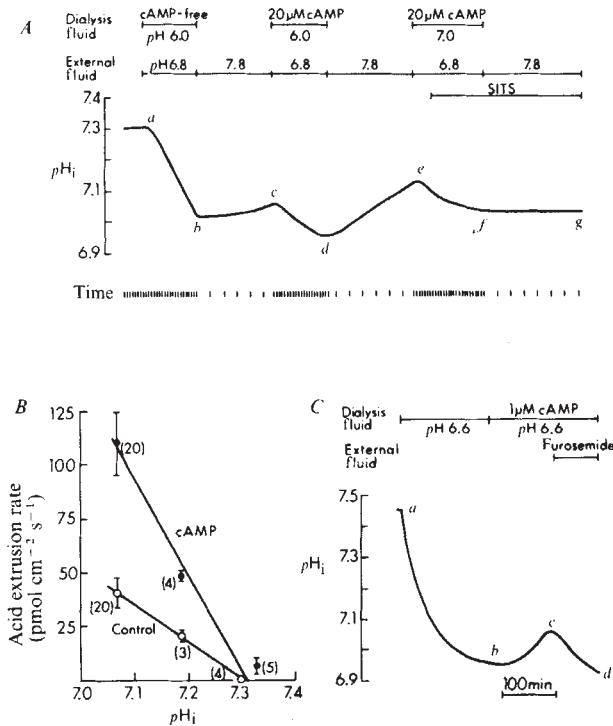


Fig. 1 A, Effect of cyclic AMP (cAMP) on acid extrusion. The pH_i was lowered by dialysing, first with cyclic AMP-free (segment *ab*) and then cyclic AMP-containing (*cd*, *ef*) fluid (50 mM HEPES buffer). On halting dialysis, the subsequent recovery of pH_i , which reflects acid extrusion, was slow in the absence of cyclic AMP (*bc*), but much faster in its presence (*de*). SITS (0.5 mM) blocked recovery, even with cyclic AMP (*fg*). Membrane potential (V_m) was -49 mV at the onset of the experiment and -48 mV at the end. The speed of the strip-chart recorder was increased fivefold during the periods of pH_i recovery; each hash mark represents 2.5 min. B, Effect of cyclic AMP on the acid extrusion rate at various values of pH_i . Data were derived from experiments similar to that above. Values of the acid extrusion rate were calculated from observed dpH/dt (during pH_i recovery) and measured intracellular buffering power, assuming that the fibre is a cylinder. Vertical bars indicate one standard error; number of experiments given in parentheses. Lines through points represent least-square fits. C, Effect of cyclic AMP on pH_i in a continuously dialysed fibre ($pH_o = 7.8$). During dialysis, pH_i is determined by the balance between introduction of acid by dialysate (pH 6.6, 50 mM PIPES buffer) and acid extrusion by the pH_i -regulating system of the cell. Addition of 1 μ M cyclic AMP to the dialysate causes a 0.1 increase in steady-state pH_i (compare *b* and *c*), reflecting a stimulation of acid extrusion, which is blocked by the addition of 0.6 mM furosemide (Hoechst) to the external fluid (*cd*). $V_m = -48$ mV. Unless otherwise indicated, artificial sea water contained (in mM): 418 NaCl, 10 KCl, 11 $CaCl_2$, 32 $MgCl_2$ and 30 HEPES, and was gassed with 100% O_2 . Although this solution was nominally HCO_3^- -free, $[HCO_3^-]_o$ in the vicinity of the muscle fibre was probably ~ 1 mM (W.F.B., McCormick and A.R., unpublished). Dialysis fluid contained (in mM): 30 KCl, 160 K glutamate, 20 Na glutamate, 4 $MgSO_4$, 550 mannitol, 0.5 EGTA, 0.5 phenol red and 50 mM HEPES or PIPES. Temperature was 22 $^{\circ}C$.

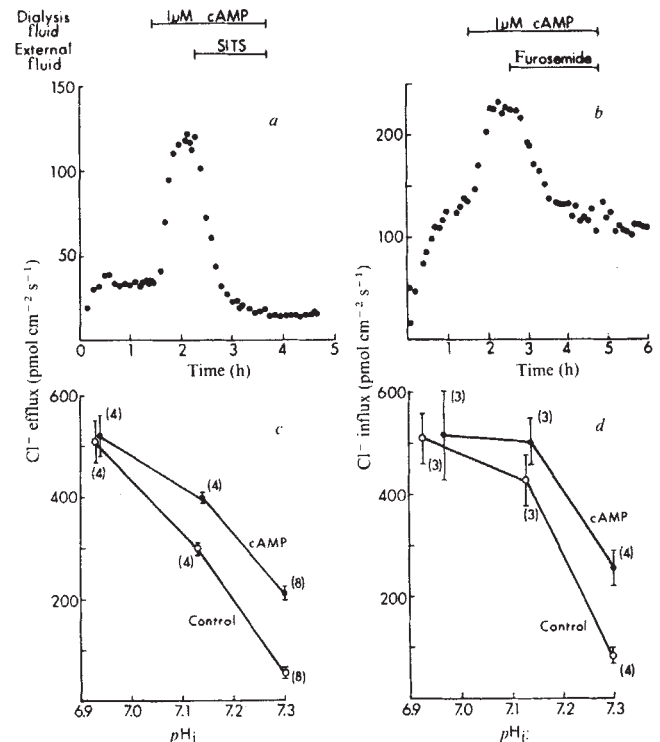


Fig. 2 a, Effect of cyclic AMP on Cl^- efflux. At time zero, flow of isotope-containing dialysis fluid, pH 7.3 (50 mM HEPES buffer) was begun. After Cl^- efflux had reached a steady state, 1 μ M cyclic AMP was added to the dialysis fluid, causing an increase in Cl^- efflux. Subsequent addition of 0.5 mM SITS to the external fluid reduced Cl^- efflux to slightly below control values. V_m was -53 mV at the outset and -49 mV at the end of the experiment. b, Effect of cyclic AMP on Cl^- influx. After Cl^- influx had reached a steady state, addition of 1 μ M cyclic AMP to the dialysis fluid caused an increase in Cl^- influx which was then reversed by the presence of 0.6 mM furosemide in the external fluid. V_m was -51 mV and -47 mV at the beginning and end of the experiment, respectively. c, d, Effect of cyclic AMP on Cl^- efflux and influx at various values of pH_i . Experiments were similar to those above. Vertical bars represent one standard error; number of experiments is given in parentheses. Lines through points drawn by hand. Actual values of pH_i were verified with pH microelectrodes (see Fig. 1C) in separate experiments; 4–5 determinations of pH_i were made for each pH of the dialysis fluid; s.e. = ± 0.03 – 0.04 .

of cyclic AMP on the dependence of acid extrusion rate on pH_i . Although cyclic AMP had little effect at the normal pH_i of 7.3, it greatly increased acid extrusion rate at low pH_i . In these experiments, a high (20 μ M) dialysate level of cyclic AMP was used to maintain adequate cytoplasmic levels after dialysis was halted. Even so, the stimulatory effects of the nucleotide faded within 10–20 min unless 3-isobutyl-1-methylxanthine, an inhibitor of cyclic AMP hydrolysis, was added. However, even 1 μ M cyclic AMP was able to increase acid extrusion rate when dialysis was continuous, as shown in Fig. 1C. Here, dialysis with fluid at pH 6.6 caused pH_i to fall (Fig. 1C, *ab*) until the entrance of acid by dialysis was balanced by acid extrusion (Fig. 1C, *b*). When 1 μ M cyclic AMP was now added to the dialysate, pH_i rose by 0.1 (Fig. 1C, *bc*), reflecting stimulation of the acid extrusion rate. Exposure to the diuretic furosemide (0.6 mM), an inhibitor of anion transport¹⁰, caused a decline in pH_i (Fig. 1C, *cd*), indicating inhibition of acid extrusion.

Evidence that acid extrusion involves an exchange of external HCO_3^- for internal Cl^- (refs 1, 2), and the effect of cyclic AMP on acid extrusion, suggested that cyclic AMP would stimulate Cl^- efflux. Figure 2a and b shows that dialysis with 1 μ M cyclic AMP stimulated not only Cl^- efflux, but also its influx. These effects could be blocked by either SITS or furosemide. Lowering pH_i stimulated efflux and influx (as it did acid extrusion rate),

Table 1 Dependence of Cl^- influx and rate of acid extrusion on $[\text{HCO}_3^-]_o$ at different values of pH_i

pH_i	Cl^- influx ($\text{pmol cm}^{-2} \text{s}^{-1}$)		Acid extrusion rate ($\text{pmol cm}^{-2} \text{s}^{-1}$)	
	$[\text{HCO}_3^-]_o$ = 0	$[\text{HCO}_3^-]_o$ = 6 mM	$[\text{HCO}_3^-]_o$ = 0	$[\text{HCO}_3^-]_o$ = 6 mM
7.30	51 ± 2 (3)	48 ± 1 (3)	0	12 ± 8 (6)
7.13	455 ± 44 (3)	310 ± 23 (3)	32 ± 8 (4)	171 ± 32 (6)
6.93	512 ± 46 (3)	351 ± 44 (3)	101 ± 18 (4)	436 ± 110 (3)

External solutions ($\text{pH}_o = 7.8$) contained either nominally 0 mM HCO_3^- (gassed with 100% O_2) or 6 mM HCO_3^- (gassed with 0.4% CO_2 , balance O_2); all solutions contained 30 mM HEPES. In acid extrusion experiments, fibres were acid loaded using the NH_4Cl prepulse technique⁸. The values are means ± s.e.; numbers of experiments in parentheses.

and these responses were further enhanced by cyclic AMP (Fig. 2c, d). Cyclic GMP did not significantly alter Cl^- efflux or influx, or the acid extrusion rate.

At pH_i 7.3, cyclic AMP stimulated both unidirectional Cl^- fluxes (Fig. 2c, d), whereas it had little effect on acid extrusion (Fig. 1B), that is, net HCO_3^- influx. As these increases in Cl^- efflux and influx are of similar size ($\sim 160 \text{ pmol cm}^{-2} \text{s}^{-1}$), it is likely that, at pH_i 7.3, cyclic AMP enhances a Cl^- - Cl^- exchange. The existence of a Cl^- - Cl^- exchange at pH_i 7.3 is supported by the requirement of Cl^- influx for internal Cl^- (J.M.R. and M.S.B., unpublished), and by our observation that, with or without cyclic AMP, Cl^- efflux and influx were inhibited to a similar extent by either SITS or furosemide. Our data indicate that reducing pH_i below 7.3 enhances an existing Cl^- - Cl^- exchange (Fig. 2c, d) and, in addition, initiates a HCO_3^- - Cl^- exchange, that is, acid extrusion (Fig. 1B). Both these exchanges were stimulated by cyclic AMP. The common sensitivity, at low pH_i , of the Cl^- - Cl^- and HCO_3^- - Cl^- exchanges to cyclic AMP, SITS and furosemide suggests that both exchanges are mediated by the same carrier. If this is the case, then HCO_3^- and Cl^- might compete for entry. This expectation is confirmed in Table 1. At pH_i 7.3, when the acid extrusion rate was zero and the exchange mechanism was in the Cl^- - Cl^- mode, increasing $[\text{HCO}_3^-]_o$ had little effect on either acid extrusion rate or Cl^- influx. However, at lower values of pH_i , raising $[\text{HCO}_3^-]_o$ not only increased the acid extrusion rate, but also decreased Cl^- influx. We suggest that pH_i regulation in barnacle muscle is accomplished by an anion exchange mechanism whose rate is enhanced by cyclic AMP and by decreases in pH_i . At pH_i 7.3, this exchange operates at a low rate in the Cl^- - Cl^- mode. At lower pH_i , the rate of exchange increases, and simultaneously the mode of exchange shifts from Cl^- - Cl^- to HCO_3^- - Cl^- . Cyclic AMP increases the rate of anion exchange in either mode, thereby promoting the fluxes of both HCO_3^- and Cl^- .

Our observations concerning the effects of cyclic AMP on pH_i regulation may have implications for any cell subjected to acid loads. Mammalian cells are of particular interest because their high metabolic rate and presumed large HCO_3^- efflux represent a sustained acid load, comparable with that in the acid-dialysed barnacle muscle fibre of Fig. 1C. Thus, hormones which increase cyclic AMP levels might raise pH_i , and thereby affect a variety of processes, with the pH_i changes serving as a third messenger.

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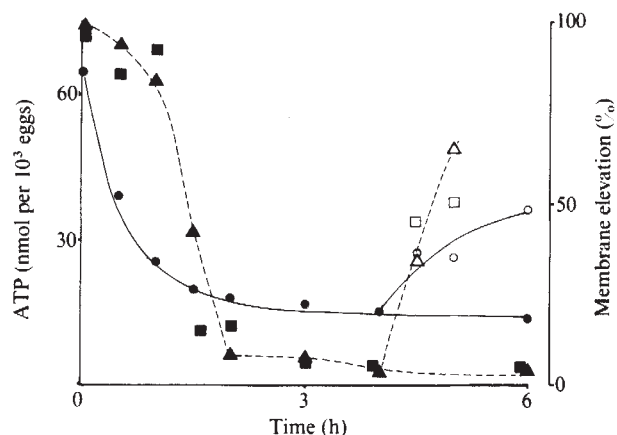
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Influence of ATP and calcium on the cortical reaction in sea urchin eggs

THE prevention of polyspermy in the sea urchin egg is a two-stage process: a rapid reduction in the chances of refertilisation—the ‘fast block’^{1,2}, which seems to be electrical in nature³—and a slower block that coincides with elevation of a morphological barrier, the fertilisation membrane. The fast block has many features in common with the action potential of excitable tissues, while elevation of the fertilisation membrane seems to involve a classical exocytotic reaction similar to that found at nerve terminals. Lying immediately beneath the plasma membrane of unfertilised eggs is an array of vesicles called cortical granules each about 1 μm in diameter. Shortly after fertilisation the contents of these granules are expelled, the granule membrane becoming incorporated into the plasma membrane of the egg. The trigger for initiating the cortical reaction seems to be a rise in intracellular ionised calcium. Thus granule release can also be promoted by the ionophore A23187 (ref. 4) and measurements in aequorin-loaded fish and sea urchin eggs^{5,6} show that exocytosis brought about by both sperm

Fig. 1 Influence of cyanide on the cortical reaction and level of ATP in eggs of *Echinus esculentus* at 16 °C. ●, ○ ATP content of eggs treated at zero time with 5 mM cyanide in artificial seawater containing (mM) NaCl, 465; MgCl_2 , 55; CaCl_2 , 11; KCl, 10; NaHCO_3 , 2.5 pH 7.8. ATP was assayed fluorimetrically⁷. Fertilisation membrane elevation in response to sperm (▲, △) and the ionophore A23187 (20 $\mu\text{g ml}^{-1}$) (■, □) is also shown. After 4 h cyanide was removed from an aliquot of poisoned eggs by thorough washing (○, △, □).



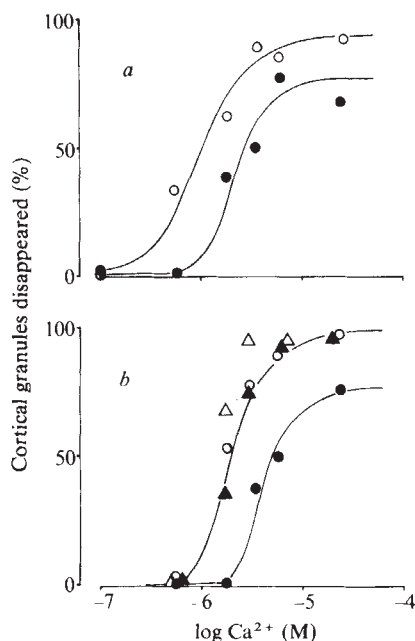


Fig. 2 Dependence of the cortical reaction in broken eggs of *Echinus esculentus* on ionised calcium at 16 °C. *a*, Eggs subjected to a high voltage discharge (1 kV cm^{-1} , $\tau = 50 \text{ } \mu\text{s}$)¹⁰ in a medium containing (mM): potassium glutamate, 250; glycine, 500; NaCl, 10; MgCl_2 , 10; HEPES 10, pH 6.7. The ionised calcium concentration was controlled with 10 mM EGTA, the abscissa representing values calculated from the stability constants¹¹. Thirty seconds after shocking, the eggs were fixed with 4% formaldehyde² and the number of cortical granules scored by high-power phase contrast microscopy. $\circ$, Control eggs; $\bullet$, eggs treated for 4 h with 5 mM cyanide. *b*, eggs attached to glass or plastic with poly-L-lysine (1 mg ml^{-1}) and broken in the artificial ooplasm described in (*a*). The portion of egg cortex left on the support could be scored at 30 s for the number of cortical granules reacted. $\circ$, Control eggs; $\bullet$, cyanide-poisoned eggs. Some eggs were broken in an artificial ooplasm containing 10 mM ATP: Δ , Control eggs; $\blacktriangle$, cyanide-poisoned eggs. Each point represents a separate experiment on cortices prepared from the same batch of eggs. The reproducibility of individual observations was better than $\pm 10\%$.

and ionophore is associated with a rise in intracellular ionised calcium. The mechanism of exocytosis in any system is unknown and the cortical granule reaction in sea urchin eggs seems to provide an excellent experimental preparation for its investigation. Here we examine the influence of metabolic poisons and calcium on the cortical reaction. In the presence of ATP exocytosis is activated by micromolar concentrations of calcium; but in the nominal absence of ATP the exocytotic process slowly becomes refractory to calcium.

Eggs of *Echinus esculentus* were used and the methodology was as described previously². Figure 1 shows results obtained with intact eggs. The metabolic poison cyanide reversibly blocks cortical granule release and fertilisation membrane elevation in response to both sperm and ionophore. These effects do not result from an action of cyanide *per se* on the activating agent because control eggs elevate normal membranes when exposed to sperm or ionophore in cyanide. Essentially similar results have been obtained with dinitrophenol (DNP) (0.2 mM, pH 7.0)⁷. Also, the onset of inhibition of cortical granule release closely parallels a fall in energy-rich phosphate compounds in the egg.

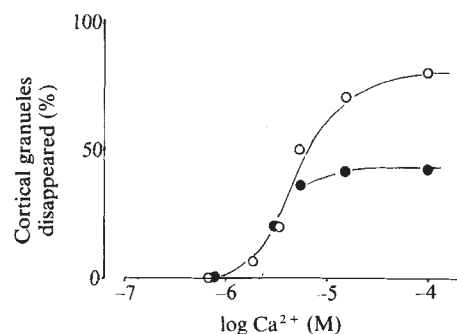
These observations on poisoned eggs can be explained in a number of ways—for example, poisoning may deplete the cell of something essential for activation, or increase the level of an inhibitor. To investigate these possibilities we examined the cortical reaction under controlled conditions using two partially broken egg preparations. In the first, rapid access to the interior of the cell was obtained by exposing suspensions of eggs to a

high-voltage discharge^{9,10}. Eggs were suspended in a medium similar to ooplasm. If Ca^{2+} is present in the medium at the time the shock is delivered, unpoisoned eggs subsequently undergo cortical granule release and raise a fertilisation membrane; but this response is not seen in EGTA. This technique is a modern version applicable to populations of cells of the 'pricking' technique used by many early workers to activate eggs. Figure 2 shows that the buffered Ca^{2+} concentration required to effect the release of half the cortical granules in unpoisoned eggs is close to $1 \text{ } \mu\text{M}$. This value is more than an order of magnitude lower than the only other reported measurement⁶. Also shown are the results of applying the high-voltage technique to fully-poisoned eggs. Rather surprisingly both cortical granule release and fertilisation membrane elevation can be fully activated; but the concentration of Ca^{2+} required to effect the release of half the granules, $3 \text{ } \mu\text{M}$, is somewhat higher than in unpoisoned eggs.

Another broken cell preparation has been described by Vacquier¹². Eggs are attached to a surface by protamine sulphate and subjected to a shearing force. The eggs break leaving small portions of egg cortex attached to the surface. These portions of cortex respond to Ca^{2+} ; but are reported to undergo granule-granule fusion. We have found that if the technique is modified by attaching jelly-free eggs either directly to plastic or to a surface coated with poly-L-lysine¹³ and the eggs broken in the medium described above, the resulting portions of egg cortex are activated in the same range of Ca^{2+} concentrations as eggs broken by shocking and the appearance of the reacted cortices is entirely consistent with exocytosis, that is, granule-plasma membrane fusion. Isolated cortices prepared from cyanide-poisoned eggs resemble 'shocked' eggs in that they need a higher Ca^{2+} concentration to activate cortical granule discharge. Addition of ATP (10 mM) to these cortices without a change in pH restores a Ca^{2+} -sensitivity close to that of unpoisoned eggs. Figure 2 also shows that inclusion of this concentration of ATP in the medium used to prepare cortices from unpoisoned eggs gives the highest apparent affinity of the cortical response for calcium which is very similar to that found in 'shocked' eggs.

The difference in apparent affinity between eggs subjected to a high-voltage discharge (Fig. 2*a*) and eggs broken (Fig. 2*b*) in Ca^{2+} -EGTA solutions containing no ATP emphasises the dependence of the Ca^{2+} -affinity of the cortical reaction on the levels of nucleotide. The high-voltage technique allows rapid access of the Ca^{2+} buffers into the egg, while maintaining sufficient integrity to retain some ATP and ATP-regenerating systems. Breaking the egg removes the cortex from the remainder of the egg and one would expect the local ATP concentration to be much lower as a consequence. Despite the lower Ca^{2+} -affinity in eggs broken in the absence of ATP, the cortical reaction is comparable to that of unpoisoned 'shocked' eggs in

Fig. 3 Dependence of cortical granule exocytosis on ATP at 14 °C. Portions of cortex stuck to glass coverslips with poly-L-lysine and isolated in a nominally Ca^{2+} -free medium containing 1 mM EGTA and 5 mM ATP were superfused with ATP-free medium for 10 min ($\circ$) or 30 min ($\bullet$) before exposure to calcium. The ordinate is the proportion of cortical granules that had disappeared 30 s after the solution had been changed to one containing calcium.



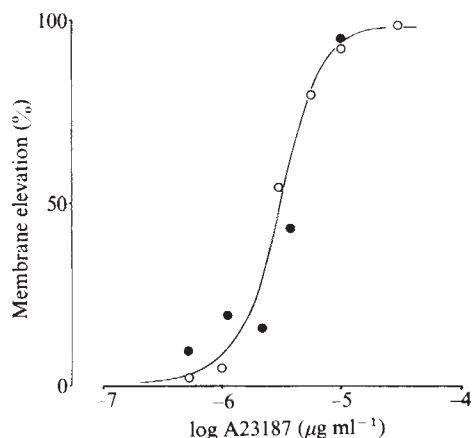


Fig. 4 Concentration-response curve for the ionophore A 23187 in ASW (○), and in Ca^{2+} -free ASW containing 5 mM EGTA (●) at 16°C. Membrane elevation was scored 3 min after addition of ionophore.

that it proceeds to completion. This contrasts with the cortical reaction of cyanide-poisoned eggs where, in addition to a shift in Ca^{2+} -affinity, some other change seems to have taken place because relatively high (100 μM) levels of Ca^{2+} are unable to effect the release of a proportion (about 15%) of the cortical granules. This effect can be mimicked in isolated cortices by superfusing for various times with nominally ATP-free solutions containing a buffered ionised calcium concentration of less than 10^{-7} M. Subsequent readmission of Ca^{2+} after exposure to ATP-free media for 10 and 30 min triggers appreciable exocytosis (Fig. 3). It is, however, noticeable that although the apparent affinity of exocytosis for Ca^{2+} remains constant, the fraction of granules released falls progressively and the unreleased granules are refractory to very high (0.5 mM) concentrations of calcium. Control cortices exposed for 30 min to Ca^{2+} -free media containing ATP subsequently respond normally to calcium. These results imply that a supply of free ATP is not necessary for exocytosis in this preparation; but the progressively increasing number of cortical granules that fail to respond to Ca^{2+} after prolonged exposure to ATP-free media suggests that tightly-bound ATP, or something derived from it, is necessary either to energise exocytosis or to confer Ca^{2+} -sensitivity on it. After prolonged exposure of isolated cortices to ATP-free media, readmission of ATP restores the high affinity of the exocytotic reaction for Ca^{2+} but fails to restore Ca^{2+} -sensitivity to the refractory granules.

Although there is a slow onset of refractoriness in the cortical response of poisoned eggs, cortices isolated from eggs in which the cortical reaction to sperm and ionophore is completely blocked still react when challenged with quite low concentrations of calcium. This is a rather surprising observation because ionophore, in particular, might be expected to promote Ca^{2+} entry across the plasma membrane until the threshold for exocytosis is reached. That this may not be so is suggested by the data in Fig. 4 which show that there is virtually no difference in the ability of ionophore to activate unpoisoned eggs in normal and Ca^{2+} -free seawater containing EGTA and measurements of $^{45}\text{Ca}^{2+}$ influx show very little stimulation by ionophore. As measurements with aequorin show that ionophore increases intracellular ionised calcium⁶, the source of this Ca^{2+} must be intracellular. In seeking an explanation for these results it should not be forgotten that poisoned eggs may contain a potent inhibitor of the cortical reaction, but another attractive possibility is that the cortical reaction in intact eggs involves the release of Ca^{2+} from an internal store. The data here might be explained if this store only remains filled in the presence of ATP. In the absence of this store, ionophore would not be able to generate a sufficient rise in ionised calcium in the vicinity of the cortical granules to initiate exocytosis. This suggestion finds

some support in the finding that isolated cortices bind $^{45}\text{Ca}^{2+}$. This binding is ATP-dependent, is much larger in cortices with intact cortical granules than in cortices that have undergone the cortical reaction, and is much reduced in the presence of the calcium ionophore A 23187.

There is another difference in the response of egg cortices prepared from unpoisoned and poisoned eggs. The response to low concentrations of Ca^{2+} in cortices prepared from unpoisoned eggs often starts at one or a few points—usually near the edge of the torn piece of cortex—and spreads in a wave or waves across the preparation. This is never seen in cortices prepared from poisoned eggs where the cortical granules respond in an apparently random fashion. This propagation reaction seen in unpoisoned cortices may also be related to the existence of a labile calcium store. Thus a rise in ionised calcium at one point may promote release of calcium from the store in a way analogous to calcium-dependent release of calcium in the sarcoplasmic reticulum¹⁴. Treatment of isolated cortices with ionophore in the presence of EGTA should deplete any membrane-bound stores of calcium and permit examination of cortical granule release in the absence of the presumed store. The cortical reaction of cortices prepared from unpoisoned eggs and treated in this way closely resembles that of cortices prepared from poisoned eggs except that addition of ATP fails to induce the high calcium sensitivity characteristic of unpoisoned eggs. These findings with ionophore-treated cortices support the concept of a store but raise the possibility that the rapid shift in affinity for calcium induced by ATP may be a property of the store and not of the exocytosis reaction itself and suggest that the existence of a store of releasable calcium may be a complicating factor in studies of calcium-dependent exocytosis in this preparation.

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Novel cytochrome *b* system in phagocytic vacuoles of human granulocytes

NEUTROPHIL granulocytes are the most numerous leukocytes and have a primary microbicidal role. They are the first cells to accumulate at sites of inflammation where they engulf, kill and digest microbes. The phagocytic process is accompanied by a burst of oxygen consumption which is important for the killing of certain bacteria, as the ability to kill bacteria is impaired in anaerobic conditions¹; in chronic granulomatous disease (CGD) this enhancement of oxygen consumption is distinctively lacking². The burst of oxygen metabolism is not inhibited by cyanide

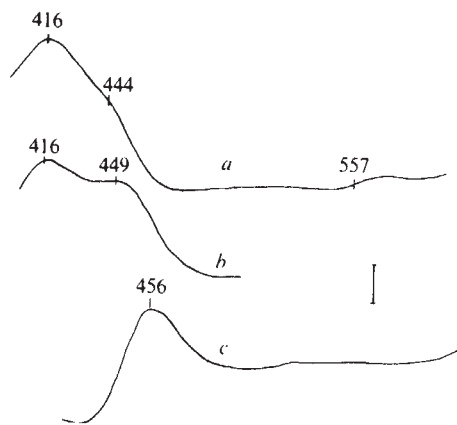


Fig. 1 Difference spectra of human neutrophils incubated with latex particles (*a*), latex particles in the presence of (2 mM) KCN (*b*) or phorbol myristate acetate (*c*). Absorbance scale = 0.028 of full scale for *a* and *b* and 0.011 for *c*. Spectra were determined in the few minutes after mixing 10^8 purified neutrophils⁵ in 3 ml of Hanks BSS with 2.5×10^9 latex particles preopsonised with human IgG (ref. 12) or with 7.5 μ g of phorbol myristate¹³ in 10 μ l DMSO.

or azide³, distinguishing it from mitochondrial respiration by these cells which contain few mitochondria and derive most of their energy from glycolysis. The subcellular localisation and mechanism of this oxidase system have been the subject of considerable investigation and controversy. We describe here a cytochrome *b* system which becomes incorporated into the phagocytic vacuoles of human neutrophils. It is distinct from the cytochrome systems of the endoplasmic reticulum and mitochondria, and abnormalities in patients with CGD implicate it as an essential component of the microbicidal oxidase system.

Human neutrophils were purified by dextran sedimentation of erythrocytes, centrifugation through a gradient of Ficoll-sodium metrizoate and hypotonic lysis of residual erythrocytes. The effect of stimuli that initiate the metabolic burst on the visible light spectrum was examined in a split-beam spectrophotometer⁴ after the addition to a suspension of purified neutrophils of latex particles opsonised with IgG or phorbol myristate acetate. This resulted in marked spectrophotometric changes which differed for the two stimuli. The latex particles produced a broad peak at about 416 nm with a shoulder at 442 nm and a minor peak at about 560 nm (Fig. 1). The 416-nm peak was reduced by the addition of KCN (2 mM) revealing the shoulder at 442 nm as a distinct peak at 449 nm. Phorbol myristate produced a peak at 456 nm with a secondary smaller peak at ~430 nm.

The possibility that these spectroscopic changes could be related to phagocytic vacuoles was then examined as this is the site where the oxidase system unites with the engulfed microbe⁵. A diaphorase enzyme system in neutrophils has been located in the plasma membrane, which invaginates to form the walls of the phagocytic vacuole and has been shown to be abnormal in CGD⁶. Phagocytic vacuoles (Fig. 2) were isolated by flotation on sucrose gradients followed by differential centrifugation. The preparation contained latex particles, most of which were enclosed in bilamellar membranes. Granule contents could be clearly seen to have been released into some of the phagosomes. Concanavalin A binds to the surface of neutrophils⁷ and a radiolabelled derivative was used as a plasma membrane marker at concentrations well below those that result in metabolic stimulation of the cell⁷. Ten per cent of the radioactive lectin became bound to the cells and 1.44% of the total activity in the homogenate was recovered with the phagosome preparation. This was found to be free of measurable contamination by endoplasmic reticulum as it contained less than 0.004% of the marker enzyme NADPH cytochrome *c* reductase⁵ present in the homogenate, and lacked spectrophotometric evidence of

cytochrome P450 (ref. 8). Complete lack of spectroscopic evidence of cytochrome oxidase indicated little mitochondrial contamination⁵.

Dithionite difference spectra of these vacuoles gave distinct peaks at 429 and 560 nm at room temperature, characteristic of a cytochrome *b* (ref. 8). These peaks were more clearly defined at slightly shorter wavelengths when examined at 77 K (Fig. 3).

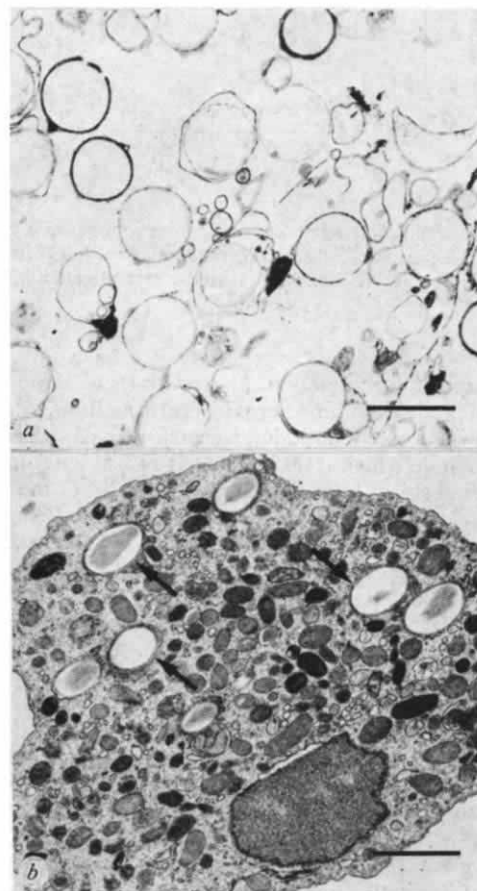


Fig. 2 Electronmicrographs of *a*, phagocytic vacuoles containing latex particles, and in some cases granule contents, within bilamellar membranes were prepared from *b*, purified human neutrophils which had phagocytosed the particles (arrows). *N*-[acetyl-³H]-acetylated concanavalin A (10 μ Ci, 16.9 Ci mmol⁻¹, Amersham) was added to 10^{10} cells from 20 donors in 50 ml RPMI (Flow Labs) containing heparin (5 IU ml⁻¹). This was centrifuged at 400g for 10 min and the pellets were suspended in 50 ml of RPMI/heparin and incubated with 5×10^{10} latex particles, opsonised with IgG (ref. 12), for 75 s at 37 °C. The cell suspension was mixed with ice-cold Hanks BSS containing 1 mmol l⁻¹ EDTA, centrifuged at 400g for 10 min, and the pellets were homogenised in 15 ml of 0.34 mol l⁻¹ sucrose in a Douce homogeniser with 100 strokes of a B pestle. The homogenate was mixed with 30 ml of 50% sucrose and 15 ml of the mixture was placed in the bottom of a 60 ml Beckman centrifuge tube and overlaid with 30 ml of 23% and then 10 ml of 11.2% sucrose and centrifuged at 100,000g for 10 h at 4°C in a Beckman L2-65B centrifuge. The material at the interface between the 11.2% and 23% sucrose was aspirated, the sucrose concentration adjusted to 0.34 mol l⁻¹ with H₂O containing 1 mM EDTA and 5 IU ml⁻¹ heparin, and centrifuged at 27,000g for 15 min in a Sorvall SS-3 centrifuge. The pellets were resuspended in 0.34 mol l⁻¹ sucrose which, like all the other sucrose solutions, contained 1 mmol l⁻¹ EDTA and 5 IU ml⁻¹ heparin. Scale bars, 1 μ m.

A carbon monoxide difference spectrum produced a small peak at 420 nm which could indicate binding of CO by the cytochrome *b*. Such a CO-binding pigment may function as an oxidase (cytochrome *o*) of a type which has not been previously described in mammalian cells⁸. Dithionite difference spectra on

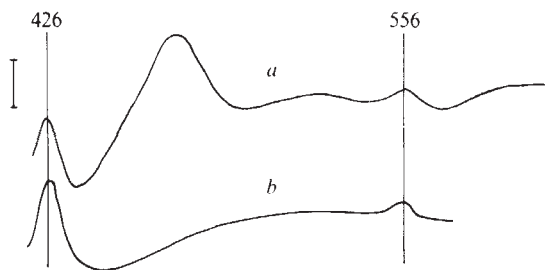
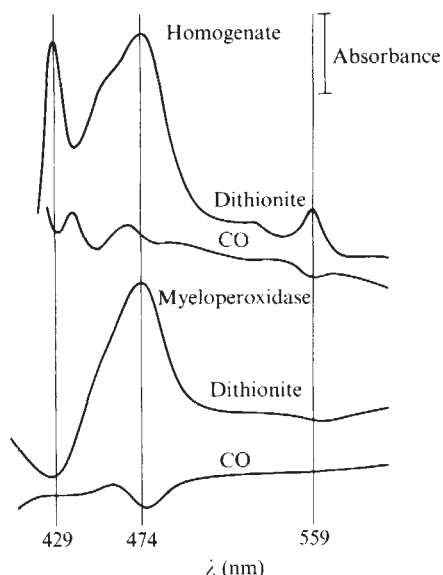


Fig. 3 Dithionite difference spectra at 77 K of a whole neutrophil homogenate (a) and a purified preparation of phagocytic vacuoles (b) (see legend to Fig. 2 for experimental details). Absorbance scale = 0.0056 and 0.0028 of full scale for a and b respectively.

the whole homogenate gave a similar pattern with, in addition, a peak at 475 nm and a minor broad peak between 510 and 540 nm. Similar difference spectra were observed in three separate homogenates each containing pooled cells from 20 normal subjects, and in 15 homogenates of cells from individuals. The 475-nm peak was identified as that of myeloperoxidase using purified human material⁹ for which the dithionite difference spectra gave a classical peak at 475 nm which was shifted slightly to 462 nm by the addition of CO (Fig. 4). There were no obvious peaks at lower wavelengths. There was no spectroscopic evidence of myeloperoxidase in the phagocytic vacuole preparation (Fig. 3) which was calculated to contain ~2.4% of the cytochrome *b* in the whole homogenate by comparing the integrated areas under the 560-nm peaks.

These studies indicate the presence of a relatively pure cytochrome *b* in the phagocytic vacuoles which is distinct from both the P450 system in the endoplasmic reticulum and the mitochondrial cytochrome system. This cytochrome seems to be a likely component of the microbicidal oxidase system and its spectrum was thus examined in neutrophil homogenates from patients with CGD. This condition is characterised by defective activity of this oxidase system³ and impaired killing of certain bacteria, leading to recurrent pyogenic infections which are often fatal. The characteristic spectrum of cytochrome *b* was missing or grossly abnormal in cell homogenates in all six of the patients that we have studied¹⁰. Half the normal level of this

Fig. 4 Dithionite and CO difference spectra of an homogenate of neutrophils from a pool of 20 normal donors and of purified human myeloperoxidase. Neutrophils were purified and homogenised in 0.34 mol l⁻¹ sucrose as described in Fig. 2, and 4 mg of protein was added to each cuvette. Absorbance scale = 0.0056 of full scale.



cytochrome was found in the two, clinically unaffected, maternal obligate heterozygote carriers¹⁰ of this sex-linked disease¹¹, indicating that the spectral changes observed in the patients were not simply a consequence of ill health.

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24, 25-Dihydroxyvitamin D is a metabolite of vitamin D essential for bone formation

THE question whether vitamin D acts directly at sites of bone formation or indirectly at sites of calcium and phosphate absorption has long been a source of argument, with no general conclusion reached. Treatment of rachitic chicks with 1-hydroxyvitamin D₃, the synthetic analogue of 1,25-dihydroxyvitamin D₃, does not prevent most of the changes seen in bone affected by rickets, in spite of normal plasma levels of calcium and inorganic phosphate (unpublished). Similar findings have been reported for man¹, suggesting that vitamin D affects bone formation directly and that the effect is mediated by a metabolite(s) other than 1,25-dihydroxyvitamin D₃. In normocalcaemic animals, the predominant dihydroxylated metabolite of vitamin D in the blood is 24, 25-dihydroxyvitamin D and, like 1,25-dihydroxyvitamin D, it is synthesised in the kidney^{2,3}; but nothing is known of its function. Improved calcium retention has been reported in anephric patients treated with 24,25-dihydroxyvitamin D₃ (ref. 4), which has been observed to accumulate preferentially in fetal bones of rat⁵ and in callus tissue formed after experimental fracture (unpublished). We now

Table 1 Body weight and plasma concentration of calcium and inorganic phosphate of chicks treated with vitamin D derivatives

Treatment	Body weight (g)	Plasma Ca (mg per 100 ml)	Plasma Pi (mg per 100 ml)
Vitamin D ₃	264.2 ± 5.3	10.9 ± 0.40	6.8 ± 0.22
24,25-Dihydroxyvitamin D ₃	266.7 ± 9.2	9.4 ± 0.45	6.3 ± 0.17
24,25-Dihydroxyvitamin-D ₃ + 1 hydroxyvitamin D ₃	289.2 ± 7.9	10.4 ± 0.22	6.9 ± 0.31
None	211.7 ± 9.0	6.3 ± 0.12	5.8 ± 0.17

All figures are mean values for six birds ± s.e.m.

Table 2 Comparison of tibial epiphyses, metaphyses and diaphyses in the different groups

24,25-Dihydroxyvitamin D ₃	1-Hydroxyvitamin D ₃ + 24,25-dihydroxyvitamin D ₃	None (-D ₃)	Vitamin D ₃	Treatment
3.3±0.3	3.4±0.3	5.0±0.4	3.5±0.2	Width of cartilage in epiphyses (mm)
25±4	26±4	35±8	28±6	Width of metaphyseal bone trabeculae (µm)
9±2	5±1	16±3	6±1	No. of osteoclasts per mm in the metaphysis
+	+	++	±	Hypertrophied osteocytes in the diaphysis
±	±	+++	±	Connective and preosteogenic tissue between trabeculae in metaphysis
+++	++	+	++	Haemopoietic bone marrow in metaphysis

Measurements were made by projecting the haematoxylin and eosin stained para-sagittal longitudinal sections of tibiae on a microscope screen. The width of the epiphyses was measured $\times 40$ and that of metaphyseal trabeculae $\times 400$. Metaphyseal osteoclasts were counted for areas 0.5×0.5 mm ($\times 400$) and calculated per mm² as described before⁸. The figures are mean values $\pm$ s.e.m. of 5–10 measurements from each chick, six chicks in each group.

report that when rachitic chicks are exposed to various therapeutic treatments with metabolites of vitamin D, the presence of 24,25-dihydroxyvitamin D is essential for the healing process.

One-day-old male chicks (*Gallus domesticus*) were depleted of vitamin D by feeding them a vitamin-D-deficient diet⁶ for 4 weeks. They were then divided into four groups and injected subcutaneously daily with, respectively, vitamin D₃; 24,25-dihydroxyvitamin D₃; 24,25-dihydroxyvitamin D₃ and 1-hydroxyvitamin D₃, or nothing but propylene glycol. After 11 d the chicks were weighed and killed, and plasma was prepared for the estimation of calcium and inorganic phosphate as before⁷ (Table 1). Proximal tibiae were removed, fixed in formalin, decalcified in formic acid and embedded in paraffin. Longitudinal 6–7-µm sections were stained with haematoxylin and eosin or with toluidine blue and examined by light microscopy. The length of the cartilaginous epiphyses, the width of the metaphyseal trabeculae and the number of metaphyseal osteoclasts were compared between groups. Other important morphological findings were graded from $\pm$ to +++ (Table 2).

As Table 1 shows, the vitamin-D-deficient chicks, compared with the vitamin-D₃-treated chicks, showed hypocalcaemia

Fig. 1 Tibial epiphysis from a vitamin-D-depleted chick. Note typical rickets; there is a wide zone of abnormally orientated chondrocytes between the proliferating zone and the zone of provisional calcification. (Haematoxylin and eosin $\times 120$.)

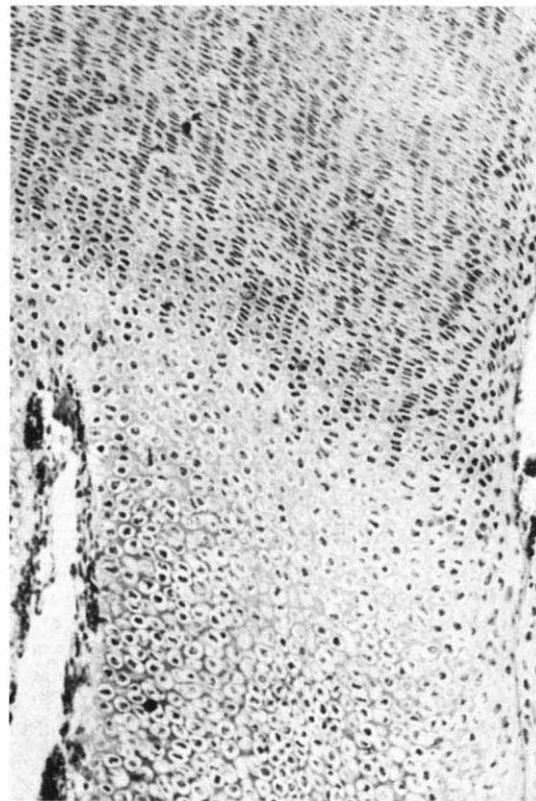


Fig. 2 Tibial epiphysis from a chick treated with 24,25-dihydroxyvitamin D₃ and 1-hydroxyvitamin D₃. Note normal arrangement of the proliferating, maturing and hypertrophic zones. (Haematoxylin and eosin $\times 120$.)

($P < 0.01$) and hypophosphataemia ($P < 0.01$) associated with growth retardation ($P < 0.05$). 24,25 Dihydroxyvitamin-D₃-treated birds grew as well as those treated with vitamin-D₃, but were unable to maintain normal plasma levels of calcium ($P < 0.05$). Only birds treated with both 24,25-dihydroxyvitamin D₃ and 1-hydroxyvitamin D₃ maintained normal plasma levels of both calcium and inorganic phosphate, and their growth was apparently better than that of vitamin-D₃-treated birds ($P < 0.05$).

The tibial epiphyses in all birds were still cartilaginous. In the controls (vitamin-D₃-treated birds) the different zones of the epiphyses, which were 3.5 ± 0.2 mm long (Table 2) were well defined. There was a straight demarcation line between the proliferating, maturing and hypertrophic zones. Metaphyseal trabeculae were parallel to the long axis of the tibiae. There were only a few osteoclasts (Table 2).

In the vitamin-D-deficient chicks the tibial epiphyses were longer— 5.0 ± 0.4 mm (Table 2). The zone of maturation was wide and disorganised, and the demarcation line between the zones of maturation and of hypertrophy was poorly defined (Fig. 1). In the metaphysis these were cartilaginous bars surrounded

by osteoid, and numerous osteoclasts were found around the metaphyseal trabeculae (Table 2). The spaces between metaphyseal trabeculae were filled with a large amount of connective and preosteogenic tissue. In the diaphysis these were many hypertrophied osteocytes in wide lacunae.

Treatment with 1-hydroxyvitamin D₃ together with 24,25-dihydroxyvitamin D₃ completely cured the rickets, and the bones were indistinguishable from the control (Fig. 2, Table 2). Treatment with 24,25-dihydroxyvitamin D₃ also prevented rachitic bone lesions. Epiphyses were of the same length and morphology as in vitamin-D₃-treated chicks (Table 2, Fig. 3). In the metaphysis, however, there were fewer trabeculae compared to controls, but the diaphysis appeared normal in width. Abundant haemopoietic tissue was found between the metaphyseal trabeculae, and the number of metaphyseal osteoclasts was slightly greater (Table 2). Osteocytic hypertrophy was also seen in some diaphyseal trabeculae (Fig. 4). These findings, not characteristic of rickets, seemed to be a reflection of the lower plasma calcium levels.

Our study shows that in young growing chicks a combined treatment of the two renal dihydroxylated metabolites of vitamin D can prevent the bone changes seen in rickets, and that the bones formed in such birds are indistinguishable from those formed in birds treated with vitamin D₃. 24,25-Dihydroxyvitamin D₃ alone seems to be sufficient to prevent rachitic bone lesions, at least those affecting cartilage, but probably because of low levels of plasma calcium in our chicks, we saw some changes in the treated bones (fewer metaphyseal trabeculae than normal, together with osteocytic hypertrophy). Based on these findings and the observation that treatment with 1-hydroxyvitamin D₃ as the only source of vitamin D in chicks cannot prevent rachitic changes in bones in spite of normal levels of plasma calcium and inorganic phosphate (unpublished), we propose a function for 1,25-dihydroxyvitamin D and for 24,25-dihydroxyvitamin D₃; we suggest that 1,25-dihydroxyvitamin D₃ increases the calcium and phosphate levels of the extracel-

Fig. 3 Tibial epiphysis from a chick treated with 24,25-dihydroxyvitamin D₃. Note normal arrangement of proliferating, maturing and hypertrophic zones. (Haematoxylin and eosin $\times 120$.)

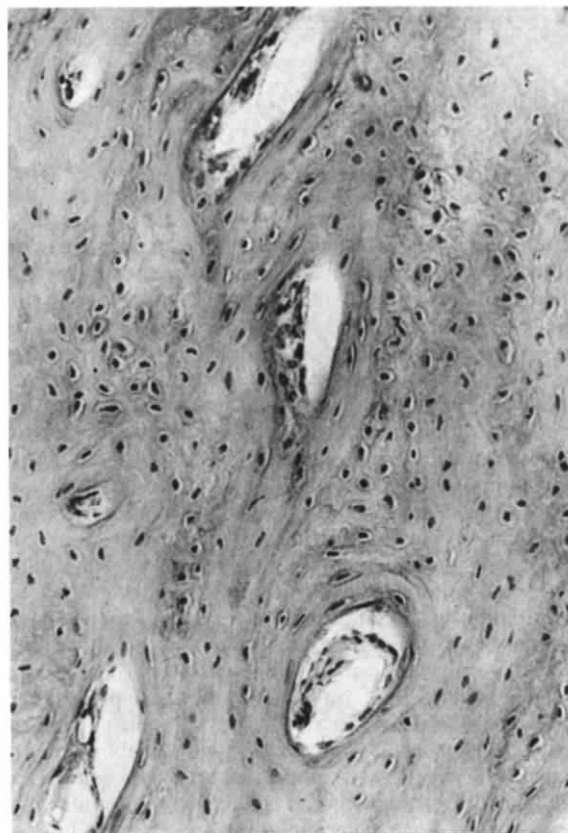
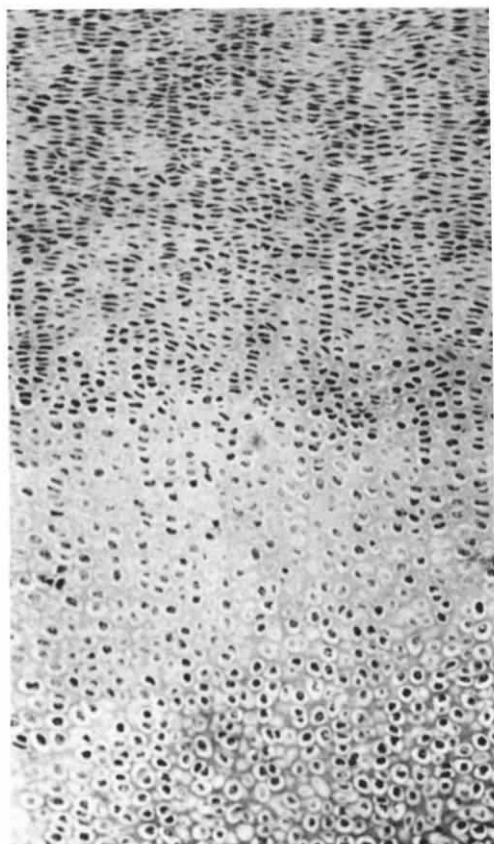


Fig. 4 Tibial diaphyseal trabecula to show slight osteocytic hypertrophy. This chick was treated with 24,25-dihydroxyvitamin D₃. (Haematoxylin and eosin $\times 250$.)

lular fluid to a supersaturated state in which conditions, in the presence of 24,25-dihydroxyvitamin D₃ which probably acts directly on bone, mineralisation of bone matrix will occur. The possibility that the beneficial effect of 24,25-dihydroxyvitamin D₃ in the presence of 1-hydroxyvitamin D₃ is due to the formation of 1,24,25-trihydroxyvitamin D₃ was ruled out, as it was found that in similar conditions, using tritiated 24,25-dihydroxyvitamin D₃, 1,24,25-trihydroxyvitamin D₃ could not be detected in intestine kidney or bone. The exact role that 24,25-dihydroxyvitamin D₃ plays in this process remains to be established.

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Ectopic dendritic growth in mature pyramidal neurones in human ganglioside storage disease

PYRAMIDAL CELLS are highly differentiated cortical neurones whose morphophysiological properties derive largely from dendritic systems that provide most of the postsynaptic sites for excitatory and inhibitory synaptic inputs¹. As pyramidal cell function is closely linked to dendritic geometry^{2,3} factors influencing dendritic growth determine to a large extent connectivity patterns in cortical neuronal organisations⁴⁻⁶. Although various experimental manipulations hinder attainment of normal dendritic geometry⁷ no factors or conditions are known to induce dendritic growth and differentiation in intact, mature cortical neurones. I present here morphological evidence that in human ganglioside storage disease pyramidal neurones, having completed an initial period of normal dendritic growth, undergo a second phase of dendritic growth and differentiation at aberrant sites. I have referred to this in another context as ectopic dendrogenesis⁸.

Observations were made initially on cortical pyramidal neurones from a 14-month-old child who presented with seizures and mild neurobehavioural retardation⁹. The diagnosis of G_{M2} -gangliosidosis, AB variant, was established by biochemical analysis of brain biopsy tissue¹⁰. Golgi studies at the time of the diagnostic biopsy revealed two features of pyramidal neurone morphology. These features are apparent in Fig. 1A; Fig. 1B shows postmortem findings. The neurone in Fig. 1A has well developed and normal appearing dendritic systems as judged by other developmental data on neurones of immature

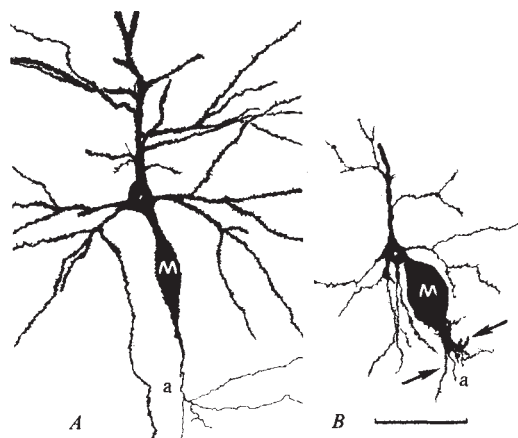


Fig. 1 Changes in abnormal growth characteristics of cortical pyramidal neurones in human ganglioside storage disease, G_{M2} -gangliosidosis (AB variant). Camera lucida drawings of Golgi preparations. *A*, Early stage; onset of seizures and developmental retardation. Neurone as seen in brain biopsy tissue. Reproduced by permission⁹. Dendrites truncated for purposes of illustration. Scale bar 75 μ m. *B*, Terminal stage neurobehavioural deterioration, 30 months after observations in (*A*), postmortem tissue. Compare relative size of cell bodies (asterisks) and meganeurites (M) at the two stages. Atrophy of the normal dendritic system and robust growth of aberrant dendrites from the meganeurite (at arrows) occur late in the gangliosidosis. *a*, Axon. (*A*) is reproduced from ref. 9.

human cerebral cortex¹¹. The most significant finding reported originally was the presence of large, fusiform spine-bearing structures, meganeurites (M in Fig. 1A) interposed between the cell body and initial portion of the axon. Meganeurites have

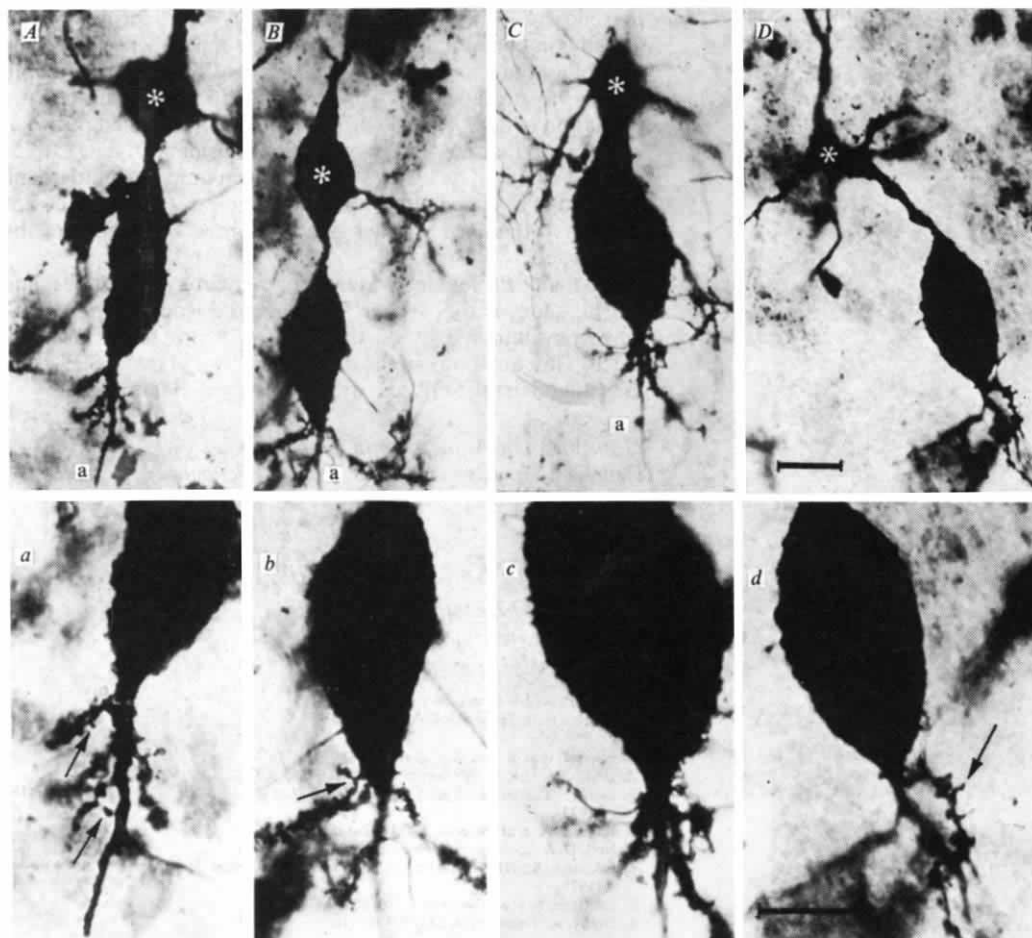


Fig. 2 *A-D*, Examples of medium sized pyramidal neurones at the terminal stage of G_{M2} -gangliosidosis (AB variant) in a 44-month-old child. Golgi preparations of postmortem brain tissue. Cell bodies (asterisks) give rise to variable processes that become massive fusiform or oval structures, meganeurites. Most meganeurites are covered with spines, shown more clearly at higher magnification (*a-d*). Dendrites with dendritic spines (at arrows in *a*, *b* and *d*) arise from the meganeurites. *a*, Axon. Scale bars, 20 μ m.

since been found in several human ganglioside storage disorders⁹ and in feline G_{M1} -gangliosidosis¹².

Between initial diagnosis and death, 30 months later, the child's clinical condition deteriorated in a manner similar to that seen in classical Tay-Sachs disease. Examination of Golgi preparations of postmortem brain tissue (Fig. 1B) showed striking changes in pyramidal cells from the first observations 30 months earlier (Fig. 1A). Extraordinary growth of meganeurites and atrophy of perisomatic dendritic systems are evident as described in classical Tay-Sachs disease⁹. A significant finding is the outgrowth from the meganeurite of robust dendrites bristling with dendritic spines (Fig. 1B, at arrows). Further examples of small and medium pyramidal neurones with prominent dendrites arising from meganeurites are shown in Fig. 2. Several meganeurite-related dendrites in Fig. 2(a-d) exhibit a variety of spine shapes characteristic of normal dendritic systems of mature pyramidal neurones¹³.

Golgi impregnations of cortical pyramidal neurones of post-mortem tissue establish that the aberrant structures designated meganeurites give rise to large spine-covered processes that are identical in every respect to spiny dendrites. Since at an early stage in the course of the ganglioside storage disease studied here meganeurites are prominent but devoid of neurites or dendrites, it follows that growth of meganeurite related dendrites occurred after the period of initial normal dendritic development. The finding that meganeurites serve as 'pseudosomas' for the new dendritic systems confirms previous indications that meganeurites continue to exhibit embryonic growth characteristics that are not expressed by the rest of the ganglioside-laden neurone^{9,12,14}. The mechanism by which abnormal ganglioside metabolism signals the genetic machinery of the neurone to synthesise spine-rich dendritic-like membrane for incorporation into meganeurites and related dendrites is unknown. The unprecedented findings here of aberrant dendritic growth induction in otherwise mature human cortical pyramidal neurones suggests that the pursuit of factors associated with abnormal ganglioside metabolism will shed light on the developmental mechanisms of neurone shape regulation and dendritic membrane distribution.

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α - And β -adrenoreceptor blockers have opposite effects on energy metabolism of the central auditory system

THE autoradiographic ^{14}C -deoxyglucose assay has made it possible to measure the local rates of glucose utilisation (LCGU) simultaneously in all the macroscopic components of the central nervous system¹. Because of the close relationship between local cerebral functional and metabolic activities, the method has proved useful in mapping the distribution of alterations in cerebral functional activity in experimental physiological states², and to identify components of cerebral dopaminergic systems in studies with the dopamine agonists, apomorphine³ and amphetamine⁴. Noradrenergic systems, including both α - and β -adrenergic receptors, have been identified in brain and implicated in various behavioural and affective states in man and animals^{5,6}. Drugs with α - or β -adrenergic blocking actions exhibit effects on mood and behaviour^{5,6}. Propranolol, a β -adrenergic blocking agent, has, in fact, been used as a therapeutic agent in the control of psychotic symptoms^{7,8}. In an attempt to localise the regions of the brain in which functional and/or metabolic activities are altered by adrenergic blockade, we have used the ^{14}C -deoxyglucose method to study the effects of the β -adrenergic blocker, DL-propranolol, and the α -adrenergic blocker, phentolamine. The results demonstrate strikingly opposite effects of these drugs in the auditory system of the rat.

Normal male Sprague-Dawley rats (350-400 g) were catheterised through the femoral artery and vein under halothane anaesthesia. A loose-fitting abdominal-pelvic plaster cast was applied to the animal for restraint, and the animal was allowed to recover from the effects of anaesthesia for at least 2 h. The drugs were then administered by continuous intravenous infusion. Controls were infused with normal saline at 0.04 ml per min; drug-treated animals received equivalent amounts of saline containing either DL-propranolol-HCl or phentolamine mesylate. Propranolol concentration in the saline infusion medium was adjusted to provide a rate of infusion of 0.25, 0.5 or 1.0 mg of drug per kg body weight per min; the concentration of phentolamine was adjusted to 0.25, 0.5 or 0.85 mg per kg per min. The infusions were maintained for 40 min before administration of ^{14}C -deoxyglucose and continued during the subsequent 45 min required for measurement of local cerebral glucose utilisation. The period of measurement of cerebral glucose utilisation was initiated by administration of an intravenous pulse of 125 μCi 2-deoxy-D-[1- ^{14}C]glucose (50-55 mCi mmol⁻¹) per kg body weight. Timed arterial blood samples were then drawn at predetermined intervals during the next 45 min (ref. 1). The blood samples were immediately centrifuged, and the plasma was separated and assayed for ^{14}C -deoxyglucose and glucose concentrations as previously described¹. Approximately 45 min after the pulse the animal was decapitated, and the brain was removed, frozen in Freon XII at -60 to -70 °C for sectioning and autoradiography¹. Local tissue concentrations of ^{14}C were determined by densitometric analysis of the autoradiograms¹. Local cerebral glucose utilisation was calculated from the time courses of the plasma ^{14}C -deoxyglucose and glucose concentrations and the local tissue concentrations of ^{14}C by means of the operational equation of the ^{14}C -deoxyglucose technique¹.

Administration of propranolol was associated with changes in several systemic physiological variables. Pulse rate declined; body temperature tended to fall by as much as 2 °C; and arterial P_{CO_2} and P_{O_2} tended to rise. During the administration of the propranolol all the animals showed a gradual decrease in alertness and responsiveness to tactile and auditory stimulation. Propranolol also produced dose-dependent decreases in local cerebral glucose utilisation. With increasing dosage more and more cerebral structures exhibited statistically significant

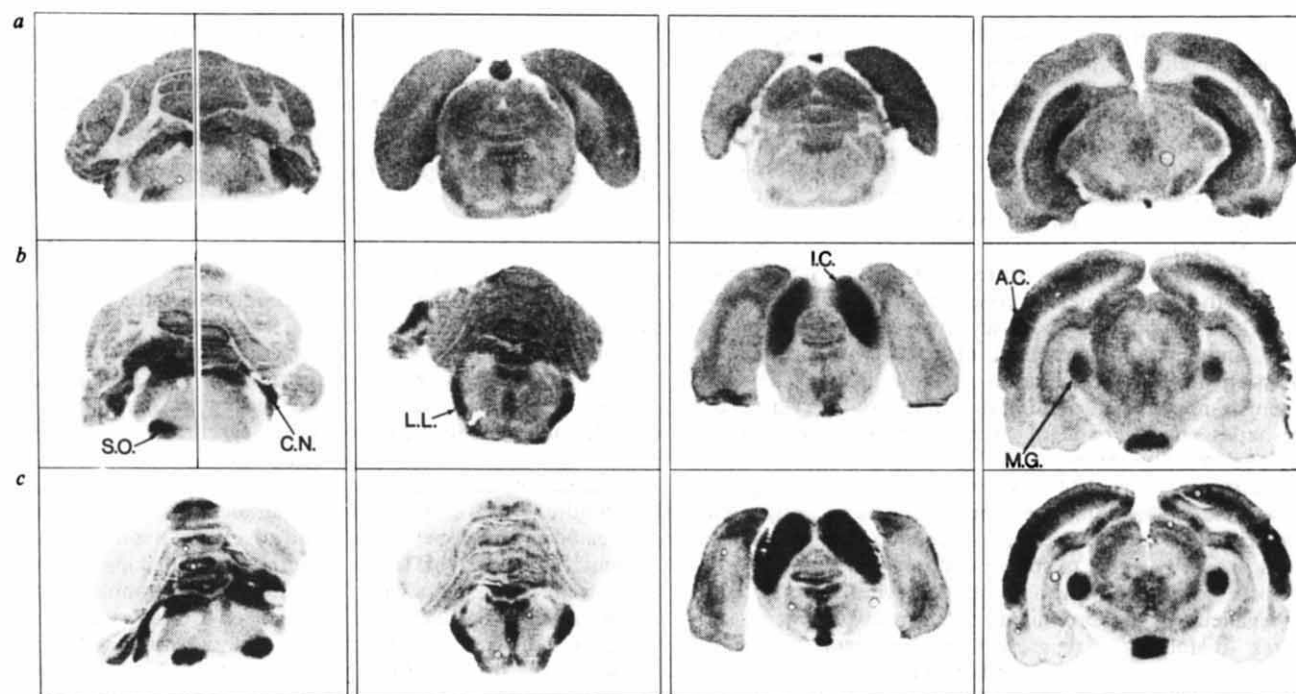


Fig. 1 Autoradiographs of brain sections from three rats illustrating effects of propranolol and phentolamine on glucose utilisation of the auditory system. The sections were at levels that include all components of the auditory system. *a*, Sections from rat given DL-propranolol-HCl 1 mg per kg per min. *b*, Sections from control rat given normal saline. *c*, Sections from rat administered phentolamine mesylate 0.85 mg per kg per min. C.N., cochlear nucleus; S.O., superior olive; L.L., nuclei of lateral lemniscus; I.C., inferior colliculus; M.G., medial geniculate body; A.C., auditory cortex.

Table 1 Effects of DL-propranolol and phentolamine on local glucose utilisation in grey matter of the conscious rat

Cerebral structures	Control (8) (μ mol per 100 g per min)	DL-Propranolol* (4) (μ mol per 100 g per min)	% Effect	Phentolamine* (6) (μ mol per 100 g per min)	% Effect
Auditory system					
Cochlear nucleus	137 $\pm$ 6	47 $\pm$ 3†	-66	168 $\pm$ 8‡	+23
Superior olivary nucleus	134 $\pm$ 7	52 $\pm$ 4†	-61	179 $\pm$ 7‡	+34
Nucleus of lateral lemniscus	104 $\pm$ 7	42 $\pm$ 2†	-60	128 $\pm$ 7†	+23
Inferior colliculus	181 $\pm$ 11	47 $\pm$ 5†	-74	228 $\pm$ 23	+26
Medial geniculate body	110 $\pm$ 4	48 $\pm$ 7†	-56	125 $\pm$ 7	+14
Auditory cortex	154 $\pm$ 5	64 $\pm$ 7†	-58	170 $\pm$ 13	+10
Miscellaneous grey matter					
Visual cortex	101 $\pm$ 3	57 $\pm$ 3†	-44	94 $\pm$ 6	- 7
Parietal cortex	104 $\pm$ 5	56 $\pm$ 4†	-46	95 $\pm$ 6	- 9
Sensory cortex	108 $\pm$ 4	55 $\pm$ 3†	-49	98 $\pm$ 6	- 9
Olfactory cortex	96 $\pm$ 3	45 $\pm$ 3†	-53	95 $\pm$ 4	- 1
Frontal cortex	105 $\pm$ 4	54 $\pm$ 5†	-49	101 $\pm$ 11	- 4
Thalamus: lateral nucleus	92 $\pm$ 4	48 $\pm$ 9†	-48	94 $\pm$ 8	+ 2
Lateral geniculate body	63 $\pm$ 2	36 $\pm$ 4†	-43	68 $\pm$ 2	+ 8
Lateral hypothalamus	44 $\pm$ 1	32 $\pm$ 3†	-27	40 $\pm$ 1	- 9
Hippocampus	70 $\pm$ 2	57 $\pm$ 3‡	-19	66 $\pm$ 3	- 6
Dentate gyrus	54 $\pm$ 2	59 $\pm$ 4	+ 9	51 $\pm$ 3	- 6
Amygdala	40 $\pm$ 2	29 $\pm$ 3‡	-28	37 $\pm$ 2	- 8
Caudate putamen	99 $\pm$ 3	56 $\pm$ 10†	-43	103 $\pm$ 8	+ 4
Nucleus accumbens	67 $\pm$ 3	42 $\pm$ 8†	-37	63 $\pm$ 3	- 6
Globus pallidus	46 $\pm$ 2	30 $\pm$ 6‡	-35	42 $\pm$ 3	- 9
Substantia nigra	52 $\pm$ 3	50 $\pm$ 7	- 4	41 $\pm$ 1†	-21
Vestibular nucleus	110 $\pm$ 3	57 $\pm$ 6‡	-48	106 $\pm$ 7	- 4
Superior colliculus	77 $\pm$ 3	40 $\pm$ 3‡	-48	74 $\pm$ 5	- 4
Pontine gray	51 $\pm$ 3	28 $\pm$ 4‡	-45	46 $\pm$ 3	-10
Cerebellar cortex	46 $\pm$ 2	29 $\pm$ 4‡	-37	41 $\pm$ 3	-11
Cerebellar nucleus	89 $\pm$ 3	48 $\pm$ 4‡	-46	88 $\pm$ 6	- 1

The values are the means $\pm$ s.e.m. obtained in the number of animals indicated in parentheses.

* DL-Propranolol-HCl: 1 mg per kg per min; phentolamine mesylate: 0.85 mg per kg per min.

† $P < 0.01$ by method of Bonferroni t statistics²³.

‡ $P < 0.05$.

reductions in glucose utilisation and to a greater degree. At the highest dosage (1 mg per kg per min) glucose utilisation was significantly depressed in all but 2 of the 26 structures examined (Table 1). Although the magnitude of the effects was substantial in many structures, the greatest depressions were in all the components of the auditory system, (cochlear nucleus, superior olive, nuclei of lateral lemniscus, inferior colliculus, medial geniculate body, and auditory cortex, Table 1). Normally, these structures have relatively high rates of glucose utilisation in the brain and, therefore, clearly stand out in the autoradiographs as areas of high optical density (Fig. 1b). Propranolol reduced the rates of metabolism of the auditory structures more than those of other structures which caused their representation in the autoradiographs to fade into the background of the other structures (Fig. 1a).

The α -adrenergic blocker phentolamine also produced systemic physiological effects. Body temperature tended to fall by as much as 3 °C, and blood pressure and pulse rate fell significantly with all doses used. Most of the animals exhibited progressive decreases in alertness and remained quiet with the eyes closed, but they responded to tactile and auditory stimuli. The penetrability of phentolamine through the blood-brain barrier has been reported to be limited⁹, but, nevertheless, the drug at the concentrations used did exert effects on the glucose utilisation of several structures of the brain. Metabolic rate tended to be reduced in most of the structures examined, but these effects were of lesser magnitude than those seen with propranolol. The most striking effects, however, were also in the components of the auditory system, but in contrast to the effects of propranolol, glucose utilisation in these structures was stimulated by phentolamine (Table 1). The effects on the auditory system were sufficiently pronounced that they could readily be visualised in the autoradiographs by the markedly increased densities in the regions corresponding to these structures (Fig. 1c).

The results of these studies demonstrate that the α - and β -adrenergic blocking agents, phentolamine and propranolol, respectively, have striking and opposite effects on glucose utilisation in the auditory system. These effects on energy metabolism seem to reflect alterations in functional activity. Furlow *et al.*¹⁰ found that doses of propranolol and phentolamine like those used in the present studies produce corresponding effects on auditory evoked responses in the rat; propranolol depresses and phentolamine enhances the amplitude of all components of the evoked auditory responses.

The stimulation of glucose utilisation by phentolamine seems to be dependent on the presence of auditory input. In rats deafened bilaterally by rupture of the tympanic membranes and occlusion of the external auditory canals with wax, phentolamine failed to increase glucose utilisation in the auditory structures significantly above the low levels seen in deafened animals. These results indicate that the effect of phentolamine is manifested only during auditory input and suggest that its effects in the auditory system may be the result of blockade of an α -adrenergic negative feedback pathway in the central auditory system. α -Adrenergic mechanisms have previously been implicated in the auditory system, and noradrenaline-containing nerve terminals have been localised in most of the structural components of the auditory system¹¹⁻¹⁶.

DL-Propranolol is not only a β -adrenergic blocking agent, but it also has membrane-stabilising properties. Experiments with sotalol, a more specific β -adrenergic blocking agent lacking membrane-stabilising properties¹⁷, indicate that it does not affect LCGU of the auditory system like DL-propranolol, but it also failed to produce the same behavioural effects, possibly because of its limited ability to cross the blood-brain barrier^{17,18}. L-Alprenolol, another specific β -adrenergic blocker, has effects similar to those seen with propranolol, but this drug also has the membrane-stabilising property. Preliminary studies with the separated isomers of propranolol indicate that they have almost equal effects on the glucose utilisation of the auditory system, despite the fact that L-propranolol is twice as potent a β -

adrenergic blocking agent as the D-isomer in the central nervous system¹⁹. Both, however, are approximately equally potent in membrane stabilisation²⁰, and these results suggest that the membrane-stabilising properties might be responsible for the effects in the auditory system.

Propranolol has recently been proposed as a therapeutic agent for treatment of psychoses^{7,8} and is particularly effective in suppressing the auditory hallucinations in schizophrenia^{7,21}. Atsmon *et al.*²² have reported a case study in which the administration of an α -adrenergic blocking agent reversed the beneficial effects of propranolol in a psychotic patient. The effects of propranolol on the auditory system observed in the present studies might be related to its effects in the control of auditory hallucinations.

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High-affinity enkephalin-degrading peptidase in brain is increased after morphine

CONSIDERABLE evidence now exists to suggest that the endogenous opioid pentapeptides Met-enkephalin (Tyr-Gly-Gly-Phe-Met) and Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu) function as neurotransmitters in the central nervous system. A correlate of this hypothesis is that a specific inactivation mechanism must operate in the vicinity of opiate receptors to turn off rapidly the enkephalin signal. Indeed recent studies have shown that the pentapeptides are subject to extremely rapid inactivation in various tissues, occurring primarily by cleavage of the Tyr-Gly amide bond¹⁻⁵. This feature accounts for the short-lasting biological activity of these peptides, contrasting with the potent activity of synthetic analogues such as (D-Ala²)-Met-enkephalinamide in which this bond is protected⁶. However, many tissues including brain contain a spectrum of peptidases with low specificities and affinities⁷ and no evidence has been yet provided for the involvement of a specific

enzyme in the regulation of enkephalineric transmission. We now report the presence of a high-affinity peptidase in a particulate fraction of mouse striatum splitting the Leu-enkephalin molecule with release of a tripeptide fragment (Tyr-Gly-Gly) and exhibiting definite substrate specificity. The marked and selective increase in the activity of this peptidase in the striatum of mice chronically treated with morphine suggests that it might be associated with enkephalineric transmission.

Enkephalin-hydrolysing activity exhibits a considerable heterogeneity in rat brain subfractions⁸. Starting from the hypothesis that a specific peptidase involved in the control of enkephalin concentration in the synaptic cleft should be membrane-bound and should exhibit a high affinity for the pentapeptides, we have compared the saturation kinetics of Leu-enkephalin-hydrolysing enzymes in a soluble and a particulate fraction from mouse striatum, a region containing a high concentration of enkephalins and opiate receptors. As shown in Fig. 1 the shape of the curves representing the fraction of ³H-Leu-enkephalin hydrolysed as a function of total substrate concentration is monophasic in the soluble fraction but clearly biphasic in the particulate fraction. In the particulate, at least two classes of enkephalin-hydrolysing activities are present; one seems to correspond to that found in the soluble fraction (IC_{50} ~

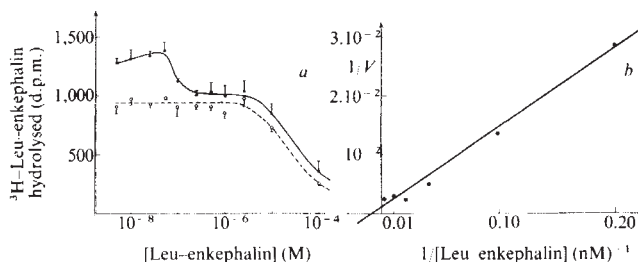


Fig. 1 ³H-Leu-enkephalin hydrolysed by particulate and soluble fractions from mouse striatum incubated in the presence of increasing concentrations of non-radioactive Leu-enkephalin. Striata of three male Swiss mice (18–20 g) were homogenised into 10 ml of 0.05 M Tris-HCl buffer pH 7.4 (3×5 s at 1,500 r.p.m. in a glass-Teflon Potter homogeniser, clearance 0.1–0.15 mm). A pellet was obtained after two successive centrifugations at 4°C ($1,000 \times 1$ min and $200,000 \times 1$ min) and the supernatant of the second centrifugation was kept as 'soluble fraction'. The pellet was resuspended using a Dounce homogeniser (clearance 20–55 μm) into 10 ml of cold buffer and centrifuged ($200,000 \times 1$ min). The resulting pellet, after a wash without resuspension with 15 ml of buffer (which was then discarded), was resuspended into 5 ml of buffer: this suspension was used as 'particulate fraction'. After a 15-min preincubation at 20°C, incubations (15 min, 20°C) of the particulate or soluble fractions were started by addition of 100 μl of buffer containing ³H-Leu-enkephalin (1 nM final concentration; the Radiochemical Centre, 41Ci mmol⁻¹, previously purified by chromatography on Porapak Q (100–120 mesh, Waters Assoc.) contained in Pasteur pipettes. The metabolites were eluted from column with 2×1 ml distilled water directly collected into scintillation vials; Leu-enkephalin was retained on the column. *a*, Effects of increasing concentrations of non-radioactive Leu-enkephalin on the formation of hydrolysis ³H-products by the particulate fraction (●) or the soluble fraction (○). *b*, Lineweaver-Burk plot of the high-affinity Leu-enkephalin hydrolysing activity (corresponding to substrate concentration below 5 μM, that is, up to the plateau) of the particulate fraction (same data as in *a*). The rate of hydrolysis (fmol per mg protein per min) was evaluated, taking into account the specific activities of the substrate. $K_m = 90$ nM, $V_{max} = 850$ fmol per mg protein per min. Means $\pm$ s.e.m. of three determinations.

Table 1 Inhibitory potencies of various compounds on the hydrolysis of ³H-Leu-enkephalin by particulate and soluble fractions of mouse striatum

	IC ₅₀ (nM) for ³ H-Leu-enkephalin hydrolysis by		Soluble fraction
	Particulate fraction		
	High affinity peptidase	Low affinity peptidase	
Leu-enkephalin	70	20,000	35,000
Met-enkephalin	350	17,000	39,400
(D-Phe ⁴)Met-enkephalin	>1,000	25,000	66,000
(L-Ala ²)Met-enkephalin	10	1,000	2,700
(D-Ala ²)Met-enkephalin	>10,000	500,000	520,000
(L-Ala ³)Met-enkephalin	97	9,700	16,500
(D-Ala ³)Met-enkephalin	>1,000	29,000	24,200
Tyr	>100,000	>100,000	>100,000
Tyr-Gly	>100,000	>100,000	>100,000
Tyr-Gly-Gly	>100,000	>100,000	>100,000
Tyr-Gly-Gly-Phe	>100,000	>100,000	>100,000
Gly-Gly-Phe-Met	1,500	>100,000	>100,000
Gly-Gly-Phe-Leu	2,100	>100,000	>100,000
Phenylmethylsulphonyl-fluoride	>100,000	>100,000	>100,000
Bacitracin	20,000	20,000	20,000
p-Chloromercuriphenyl-sulphonic acid	>100,000	1,000*	300†
Substance P	>1,000	2,000	1,800
Bradykinin	>1,000	9,000	7,500
Glutathione	>100,000	>100,000	>100,000
Carnosine	>100,000	>100,000	>100,000
LHRH	>100,000	>100,000	>100,000
TRH	>100,000	>100,000	>100,000
α-Endorphin	>5,000	54,000	56,000
β-Endorphin	>5,000	62,000	32,000
Morphine	>100,000	>100,000	>100,000
Naloxone	>100,000	>100,000	>100,000

Particulate and soluble fractions from mouse striata were prepared as described in Fig. 1. Incubations (20°C, 15 min) in the presence of 20 nM ³H-Leu-enkephalin and increasing concentrations (6–8) of the various compounds. ³H-Leu-enkephalin hydrolysis evaluated by column chromatography on Porapak Q beads (see Fig. 1). IC₅₀ values were determined by iterative analysis of the concentration-inhibition curves, analogous to that reported in Fig. 1, by a computer program based on the least squares (variance-covariance matrix)¹³. This analysis provided an accurate determination of IC₅₀ for the inhibition of the two peptidase activities in the particulate fraction in the case of a clear biphasic curve, that is, when the IC₅₀ differed by more than 50-fold. This was not the case for several compounds: (D-Phe⁴)Met-enkephalin, (D-Ala³)Met-enkephalin, bradykinin and substance P, for which a clear plateau was not present and therefore the IC₅₀ on the high-affinity peptidase could not be estimated with accuracy.

* Maximal inhibition ~70%.

† Maximal inhibition ~90%.

30 μM in both cases), whereas the other, representing 20–25% of the total hydrolysing activity at low enkephalin concentrations, is restricted to the particulate fraction. This component, represented in Lineweaver-Burk plots (Fig. 1b) exhibits a high affinity ($K_m = 90$ nM) as compared to most peptidases for which the K_m is in the range 10–100 μM (ref. 9).

In six independent experiments the presence of this high-affinity component was confirmed and the analysis of the data by a computer program (see Table 1 legend) led to mean K_m values ($\pm$ s.e.m.) of 41 ± 10 nM and of $55,000 \pm 17,000$ nM for the high- and low-affinity peptidase activities, respectively. Both peptidase activities in the particulate fraction vary linearly as a function of protein concentration or time.

The high-affinity of a class of peptidase activity found in the particulate fraction suggested that it might exhibit substrate specificity. This was indirectly investigated by determining the inhibitory potency of a variety of compounds towards the different classes of peptidase activities (Table 1). The specificity of the high-affinity peptidase activity is demonstrated by the enormous range in inhibitory potency of the various compounds tested (IC₅₀ between 10 and >100,000 nM). Although the low-affinity peptidase activities in both particulate and soluble fractions also exhibit some specificity, the range in IC₅₀ variation is much less pronounced. The main conclusions which can be drawn from the data of Table 1 can be summarised as follows: (1) Stereochemical requirements are more stringent in the case of the high-affinity peptidase as evidenced for the pair of (Ala³)-Met-enkephalin stereoisomers.

(2) The integrity of the enkephalin molecule is important for the inhibitory potency as shown by the dramatic loss of activity towards all classes of peptidases in the fragments Tyr, Tyr-Gly, Tyr-Gly-Gly and Tyr-Gly-Gly-Phe.

(3) The C-terminal moiety of the enkephalin molecule seems to be more important for inhibition of the high-affinity peptidase

Table 2 Identification by thin-layer chromatography of the radioactive compounds present after incubation of ^3H -Leu-enkephalin with a particulate fraction of mouse striatum

Solvent system	Incubation conditions	^3H -compounds (d.p.m.)			
		Before column chromatography			After column chromatography
		Tyr	Tyr-Gly-Gly	Leu-enkephalin	Tyr Tyr-Gly-Gly
(1)	Control ^(a)	1,526 ± 101	628 ± 91	14,840 ± 122	
	+Leu-enkephalin ^(a) (5 μM)	1,217 ± 171	291 ± 42**	15,524 ± 188*	
	Control	418 ± 90	465 ± 18	15,880 ± 693	500 ± 55 705 ± 58
	+Gly-Gly-Phe-Met (10 μM)	303 ± 43	118 ± 30***	16,503 ± 518	575 ± 65 265 ± 83*
(2)	Control	838 ± 110	530 ± 33	16,305 ± 478	998 ± 45 955 ± 65
	+Gly-Gly-Phe-Met (10 μM)	743 ± 15	100 ± 10***	17,545 ± 555	895 ± 15 293 ± 18***
(3)	Control				570 ± 50 660 ± 53
	+Gly-Gly-Phe-Met (10 μM)				555 ± 88 215 ± 18***

Incubation of samples (30 μg proteins per 200 μl) of the particulate fraction (15 min, 20°C) in the presence of 250 nM of purified ^3H -Leu-enkephalin and 1 μM *p*-chloromercuriphenylsulphonic acid (except in conditions (a)) and in the presence or absence of either Leu-enkephalin or Gly-Gly-Phe-Met. A 10 μl aliquot of the deproteinised supernatant or of the Porapak column eluate was applied on a TLC plate (plastic sheets Silicagel 60, Merck, thickness 0.2 mm). Solvent systems and R_f values of the reference compounds were: (1) $\text{CHCl}_3:\text{CH}_3\text{OH}:\text{AcOH}:\text{H}_2\text{O}$ (45:30:6:9); Tyr = 0.61, Tyr-Gly-Gly = 0.47, Leu-enkephalin = 0.88 (2) $\text{BuOH}:\text{AcOH}:\text{H}_2\text{O}$ (4:1:1); Tyr = 0.47, Tyr-Gly-Gly = 0.31, Leu-enkephalin = 0.58 (3) isopropanol: AcOEt : 5% AcOH (2:2:1); Tyr = 0.70, Tyr-Gly-Gly = 0.41, Leu-enkephalin = 0.80. Markers visualised with a ninhydrin spray and radioactive peaks at their R_f isolated by cutting the plates and counted by liquid scintillation (40% efficiency). Means $\pm$ s.e.m. of 3–6 determinations * $P < 0.025$. ** $P < 0.01$. *** $P < 0.001$. No other radioactive peak could be detected when the entire plate was counted by fractions of 0.5 cm. In particular no significant radioactivity was detected at the R_f s of Tyr-Gly-Gly-Phe (0.80 in system (1) and 0.45 in system (2)).

Table 3 Effect of chronic morphine treatment on ^3H -enkephalin-hydrolysing activity of particulate and soluble fractions of mouse striatum

	³ H-enkephalin-hydrolysing activity (d.p.m. per mg protein per min)			Soluble fraction
	Particulate fraction			
	High affinity peptidase	Low affinity peptidase	Total peptidase	
Controls	7,858 ± 977	33,344 ± 2,309	41,203 ± 2,620	120,990 ± 20,468
Morphine	12,521 ± 932** (+59%)	33,255 ± 1,998 ^{NS}	45,776 ± 2,042* (+12%)	125,297 ± 9,590 ^{NS}

Groups of mice were implanted under light ether anaesthesia with a morphine pellet (75 mg of base, s.c.) which was removed 3 days later, 16 h before death. Controls were sham-operated in a parallel manner. ^3H -enkephalin-hydrolysing activity measured after incubations of fractions prepared as described in Fig. 1 (15 min at 20°C) with 20 nM ^3H -Leu-enkephalin. The low-affinity hydrolysing activity of the particulate fraction represents the activity measured in the presence of 5 μM Leu-enkephalin whereas the high-affinity activity was estimated as the difference between total (in the absence of 5 μM Leu-enkephalin) and low-affinity activities. Degraded ^3H -Leu-enkephalin was measured by Porapak column chromatography. Means $\pm$ s.e.m. of 12–16 experiments on pooled striatal fractions from two mice. * $P < 0.05$. ** $P < 0.0005$. NS, not significant.

than the N-terminal moiety as shown by the much greater potency of the tetrapeptides Gly-Gly-Phe-Met or Gly-Gly-Phe-Leu when compared to the tetrapeptide Tyr-Gly-Gly-Phe; this conclusion does not hold true for the two classes of low-affinity peptidases.

(4) Among classical peptidase inhibitors, only bacitracin has significant potency towards the high-affinity peptidase, whereas *p*-chloromercuriphenylsulphonic acid, a potent inhibitor of the low-affinity peptidases, is practically inactive on the high-affinity enzyme; this indicates that the latter does not belong to the group of thiol-peptidases. The heterogeneity of enkephalin-hydrolysing activity as shown by response to thiol-blocking agents has been already suggested⁸.

(5) Structural requirements for recognition of the opiate receptors and the high-affinity peptidase are unrelated as demonstrated by the lack of potency of α - and β -endorphin as well as opiates.

From these data it seems that three compounds are useful to differentiate the high- and low-affinity peptidase activities in the particulate fraction: Leu-enkephalin, Gly-Gly-Phe-Met and *p*-chloromercuriphenylsulphonic acid. Therefore, these compounds were used as tools in the identification of the ^3H -fragments released under the action of each class of peptidases in the particulate fraction.

Thin-layer chromatography of extracts (obtained after incubation of ^3H -Leu-enkephalin with the particulate fraction) yields three radioactive peaks with R_f values closely corresponding to ^3H -Leu-enkephalin, ^3H -Tyr and ^3H -Tyr-Gly-Gly (Table 2). In contrast, only ^3H -Leu-enkephalin and ^3H -Tyr

were found after incubation of the soluble fraction. Whereas the presence of ^3H -Tyr is in agreement with a number of reports^{1–5}, ^3H -Tyr-Gly-Gly was an unexpected component. The presence of ^3H -Tyr-Gly-Gly was confirmed first by unidimensional TLC in three different solvent systems (Table 2), second by bi-dimensional co-chromatography in solvent systems (2) and (3), and third by high-performance liquid co-chromatography on a 205 U Waters apparatus (reversed phase on standard microbondapak C 18; solvent CH_3CN : 10 mM NH_4Ac buffer, 3.125:96.875; 1 ml min⁻¹; retention times Tyr = 290 s, Tyr-Gly-Gly = 350 s, Leu-enkephalin > 1 h).

The fact that Tyr-Gly-Gly was not previously identified by others as a breakdown product of enkephalin can easily be explained by its low level relative to total metabolites (~25% in our washed particulate fraction but only 2–3% in the whole homogenate as used by most workers). Nevertheless, the occurrence of a minor unidentified peak with R_f corresponding to Tyr-Gly-Gly has been reported in brain extracts², and formation of this tripeptide by cultured human endothelial cells has been reported¹⁰.

That ^3H -Tyr-Gly-Gly is specifically produced by the high-affinity peptidase is clearly demonstrated by first the marked decrease observed after incubations in the presence of 5 μM Leu-enkephalin or 10 μM Gly-Gly-Phe-Met contrasting with the unchanged ^3H -Tyr peak in the same conditions; and second the lack of effect of *p*-chloromercuriphenylsulphonic acid on ^3H -Tyr-Gly-Gly formation, contrasting with the strong reduction in the ^3H -Tyr peak (solvent system (1)). These results indicate that the high-affinity peptidase is probably a C-terminal

dipeptidase splitting the Gly-Phe bond of enkephalin, a conclusion consistent with the high inhibitory potency of the C-terminal tetrapeptide as compared to the N-terminal tetrapeptide (Table 1). As we have not yet characterised the formation of Phe-Leu or Phe-Met (because they are not labelled) we cannot exclude the possibility that ^3H -Tyr-Gly-Gly formation results from the sequential action of carboxypeptidases. However, this is unlikely because ^3H -Tyr-Gly-Gly-Phe formation was never observed in our experiments.

In mice chronically treated with morphine we observe a dramatic increase in the activity of the high-affinity peptidase in the particulate fraction (Table 3). A similar effect is also observed in rats implanted with a morphine pellet (data not shown). This effect seems to be selective because the low-affinity peptidase activity in either the particulate or the soluble fractions is unchanged. The increase in activity reflects an elevated V_{max} without significant change in K_m value (data not shown), suggesting that it corresponds to an increased number of enzyme molecules. The effect of morphine might result from a feedback mechanism operating at the target cells bearing the opiate receptors: conceivably these cells, to compensate the overstimulation of opiate receptors, could develop a state of hyposensitivity to released enkephalins, through an increased rate of inactivation of these peptides. In this respect, changes in acetylcholinesterase activity in brain have been already reported to mediate the modified responsiveness of target cells to the transmitter, developing in response to sustained changes in cholinergic transmission¹¹. Although not direct proof, the effect of morphine strongly suggests that the high-affinity peptidase is involved in the control of enkephalinergic transmission. This hypothesis is consistent with the heterogeneous distribution of this 'enkephalinase' in mouse brain regions (striatum > hypothalamus > cortex > brainstem $\approx$ hippocampus > cerebellum, with a five-fold variation between striatum and cerebellum) which is highly correlated ($r = 0.94$) with that of opiate receptors (manuscript in preparation). If true, this would imply that a new class of therapeutic agents (enkephalinase inhibitors) exhibiting opiate-like activity could be developed. In addition the increased peptidase activity after chronic morphine treatment could be involved, with other mechanisms¹², in the development of dependence on opiates.

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The ACTH 'family tree' of the rhesus monkey changes with development

THE pituitary hormone adrenocorticotrophin (ACTH) belongs to a 'family' of peptides derived from a common 'stem hormone' (Fig. 1). Depending on the way in which the stem hormone is cleaved, the various peptides are expressed and it is possible that qualitative changes in the expression of the peptide 'family tree' may be responsible for the alteration in fetal adrenal function which precedes birth¹. We now report evidence for such changes in the rhesus monkey, which has a fetal adrenal comparable with that of man.

After delivery the fetal zone of the human adrenal cortex involutes, while the definitive zone or adult cortex, consisting of a thin layer of cells covering the large fetal zone, hypertrophies.

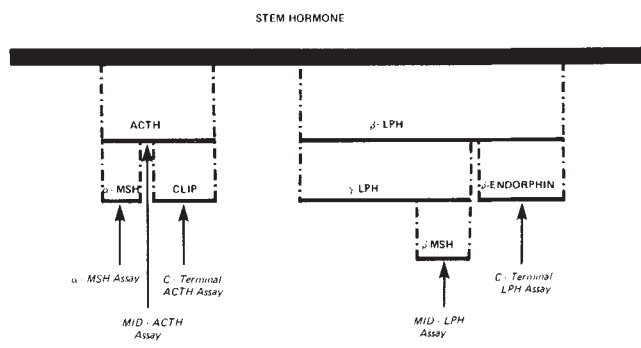


Fig. 1 The stem hormone family tree gives rise to two major branches—ACTH and β -LPH. ACTH is a 39-amino acid peptide whose first 13 amino acids are identical in sequence with α -MSH and whose 18-39 sequence is identical to CLIP. β -LPH is a 91-amino acid peptide whose first 58 amino acids are identical in sequence with γ -LPH and whose 61-91 sequence is identical to β -endorphin. The sequence 41-58 is identical to the 18-amino acid peptide β -MSH. The five radioimmunoassays are directed against different antigenic sites of this family tree.

This change may be triggered by a switch in the production of trophic hormones in the fetus, from peptides resembling α -melanocyte stimulating hormone (α -MSH) and corticotrophin-like intermediate lobe peptide (CLIP) to ACTH^{2,3}. The idea is supported by a report⁴ that α -MSH stimulated the fetal adrenal gland of the rabbit when synacthen (α -1-24 ACTH) was relatively ineffective, whereas synacthen was potent in the newborn and α -MSH was ineffective. Others have shown that although ACTH stimulated the definitive zone *in vitro* its effect on the fetal zone was inconsistent⁵. The metamorphosis of the adrenal at birth is but one aspect of the change in adrenal function. In the sheep, for example, in which there is no specific fetal zone, there are nevertheless important changes in steroid output, notably a prepartum rise of cortisol which prepares the fetus for delivery and initiates parturition⁶. As with the human, there are differences in the composition of pituitary hormones between fetus and adult¹. Unlike the human, however, it is the large molecular weight forms of ACTH which predominate in the fetal sheep. There are three such forms, two of which contain sequences common to β -lipotrophic hormone (β -LPH), γ -LPH,

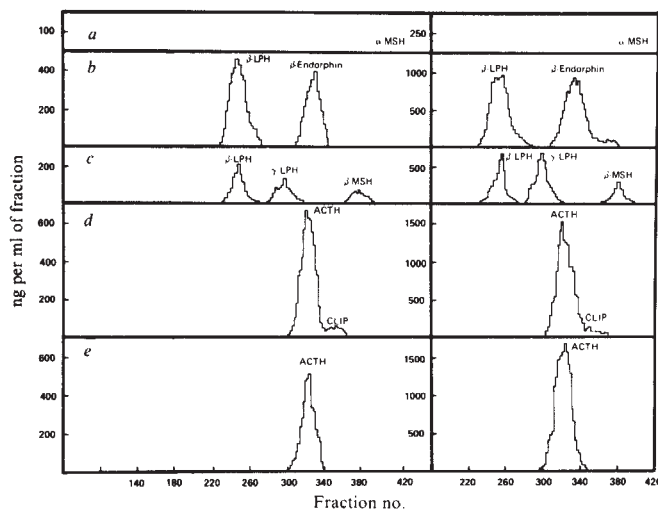


Fig. 2 Pituitary extracts from two adult monkeys (left, infant 77 days postpartum; right, mature adult) individually fractionated on Sephadex G100 superfine: alternate fractions were assayed in five systems of radioimmunoassay. *a*, α -MSH activity; *b*, C-terminal LPH; *c*, mid-LPH; *d*, C-terminal ACTH; *e*, mid-ACTH.

β -MSH, β -endorphin and possibly other biologically active peptides as well as ACTH, α -MSH and CLIP. These precursors are the 'stem hormones'.

We have used the pituitary of the rhesus monkey for further investigations because, in common with man, its adrenal gland has a fetal zone which involutes at birth. Dated pregnant monkeys were anaesthetised with 20 mg phencyclidine hydrochloride (Sennylan, Biocentrics). Fetuses were delivered by hysterotomy, their pituitaries freshly dissected, snap frozen in liquid nitrogen and stored at -70°C . Pituitaries from newborn, infant, and mature adult monkeys were freshly dissected and stored at -70°C . Pituitaries were extracted in 0.02M HCl with 10^{-3} M diisopropylphosphofluoridate, loaded on to a column (1.6×100 cm) of Sephadex G100 superfine and eluted in 1M acetic acid at 1.6 ml h^{-1} . Fractions were collected every 15 min and alternate fractions assayed in five systems of radioimmunoassay (Fig. 1) directed against different branches of the stem hormone family tree. The α -MSH assay measured only

α -MSH and was probably directed against the acetylated or amidated end groups of the molecule. The mid-ACTH assay measured ACTH and did not cross-react with α -MSH, CLIP or the LPH family. The C-terminal ACTH assay cross-reacted with CLIP as well as ACTH. The mid-LPH assay cross-reacted with β -MSH, γ -LPH and β -LPH. The C-terminal LPH assay cross-reacted with β -endorphin and β -LPH.

The patterns of peptides seen in the pituitary of an infant and a mature adult were similar (Fig. 2). The major peak in both pituitaries was ACTH with relatively insignificant amounts of α -MSH and CLIP. Further peaks of activity were associated with β -LPH, γ -LPH, β -endorphin and β -MSH. In fetal pituitaries taken at 145 days gestation and 164 days gestation, β -MSH was increased relative to β - and γ -LPH and β -endorphin increased relative to β -LPH (Fig. 3). There was a significant

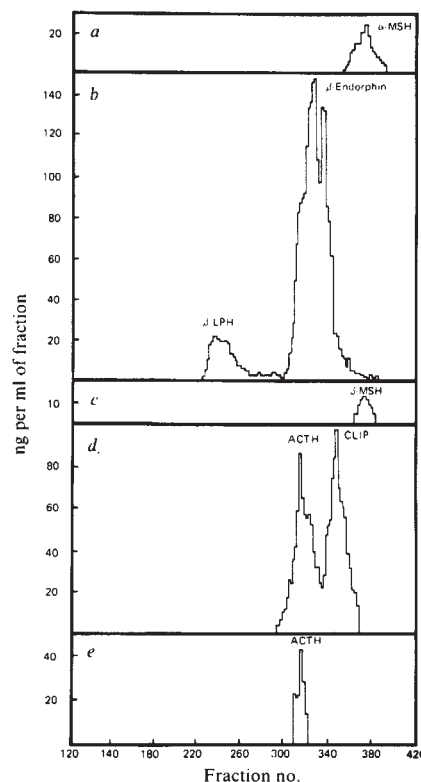
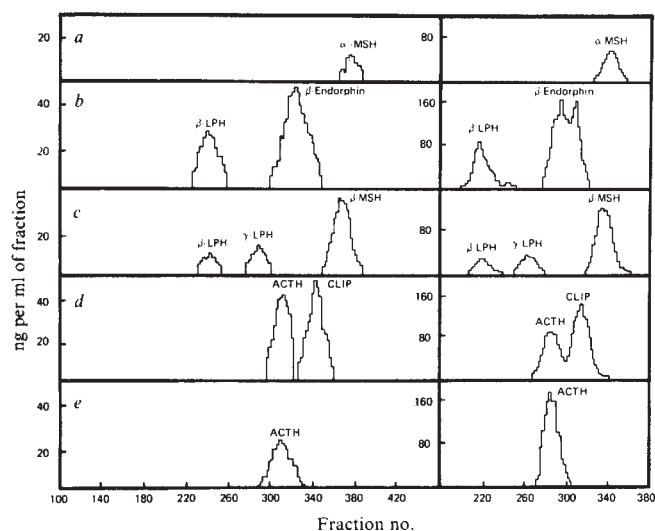


Fig. 4 Pituitary extract from a neonate (12 h postpartum) fractionated on Sephadex G100 superfine: alternate fractions were assayed in five systems of radioimmunoassay. (*a-e* as Fig. 2.)

Fig. 3 Pituitary extracts from two fetal monkeys (left, 145 days gestation; right, 164 days gestation) individually fractionated on G100 superfine: alternate fractions were assayed in five systems of radioimmunoassay. (*a-e* as Fig. 2.)



peak of α -MSH and a peak of CLIP which equalled that of ACTH. A pituitary from a neonate killed 12 h after delivery also showed substantial peaks of α -MSH and CLIP compared to ACTH (Fig. 4). However, the major peak was that of β -endorphin which dominated the peaks of β -LPH, γ -LPH and β -MSH.

The fetal and adult pituitaries did not contain substantial amounts of 'big ACTH' or stem hormone. However, small quantities were identified. An extract from a mature adult pituitary showed dominant peaks of ACTH, β -LPH, γ -LPH, β -MSH and β -endorphin, plus three peaks of large molecular weight material 'A', 'B' and 'C' (Fig. 5). Small quantities of peak A were detected by the mid-ACTH assay, peak B cross-reacted in the LPH assays and the ACTH assays, peak C only cross-reacted in the ACTH assays. The position of these three peaks corresponded to material found in abundance in the fetal sheep pituitary, where A and B were characterised as stem hormones and C as big ACTH¹.

These results reveal significant alterations of the family tree at different stages of development of the monkey. As with the

human, α -MSH and CLIP predominate in fetal life and all but disappear in the adult. Unlike the human, the pituitary of the rhesus monkey contains β -MSH with relatively greater amounts in the fetus than the adult. The levels of β -endorphin also appear more prominent in fetal life and it may consist of more than one molecular form. The overall picture is that the small molecular weight peptides α -MSH, CLIP, β -MSH and β -endorphin are characteristic of the fetal pituitary of the rhesus monkey, whereas in the adult pituitary the larger peptides ACTH, γ -LPH and β -LPH are the predominant forms.

Previously we have reported the occurrence of small amounts of a common precursor for ACTH and LPH in the human pituitary^{7,8} and such a molecule has been identified in a mouse pituitary tumour cell line^{9,10}. The stem hormones are found in abundance in the pituitary of the sheep and the fact that they can now be identified, even in small quantities, in the monkey substantiates the hypothesis that there is a family tree of biologically active peptides which derive from a common stem. The change in fetal adrenal function and morphology which precedes parturition must, therefore, be measured against the qualitative changes of the 'trophic family' as a whole.

fetus^{20,21}, it is clearly a moment of extreme physical stress and it is possible that the fetus needs to be rendered insensitive to the assault of parturition. High levels of β -endorphin at delivery may thus serve to protect the baby against a necessary but otherwise intolerable event.

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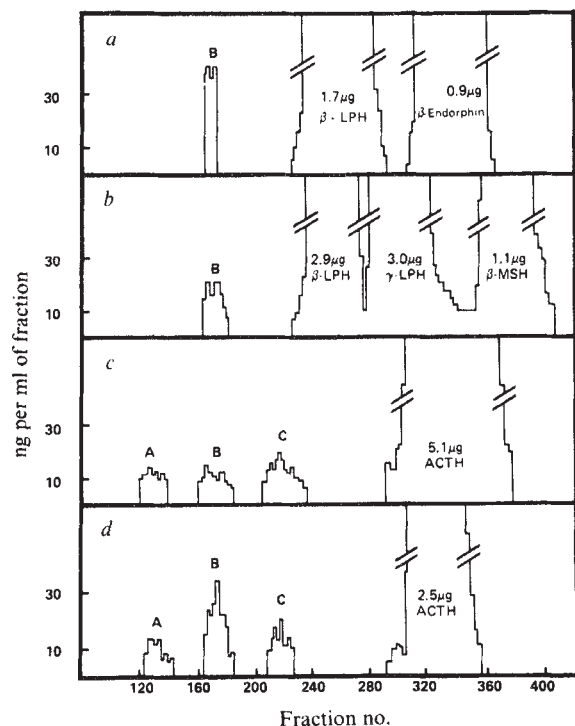


Fig. 5 Pituitary extract from a mature adult monkey fractionated on Sephadex G100 superfine. Alternate fractions were assayed in four systems of radioimmunoassay. In addition to the major peaks of ACTH, β -LPH, γ -LPH, β -endorphin and β -MSH there were three small peaks of large molecular weight material A, B and C. a, C-terminal LPH assay; b, mid-LPH; c, C-terminal ACTH; d, mid-ACTH.

There has been considerable speculation about β -endorphin since the dramatic finding that, like enkephalin¹¹, it can bind to morphine receptors¹²⁻¹⁷. The name suggests that it is endogenous morphine, but there has been little to illuminate its functional role within the mammalian neuroendocrine system. Moreover, the administration of β -endorphin has not always proved successful as an analgesic, except when given directly into the brain ventricles^{18,19}. It is an unexpected finding that there is a striking increase of β -endorphin in the pituitary of the newborn monkey (Fig. 4) and this may prove of great physiological importance. Although the moment of birth has been considered a time of intense psychological trauma for the

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Differences in fluidity between bilayer halves of tumour cell plasma membranes

SURFACE membranes of both normal and transformed cells appear to have an asymmetric distribution of phospholipids across the bilayer¹⁻⁶. The potential role of this distribution in platelet aggregation and in pathological conditions such as sickle cell anaemia and cell transformation has been investigated⁷⁻⁹. However, neither the structural nor functional significance of this asymmetrical distribution is understood at present. As the lipid composition of the two sides of the plasma membrane bilayer is different it seems possible that the 'fluidity' or other physicochemical properties are also different. It has been shown that the neutral zwitterionic lipids (phosphatidylcholine and sphingomyelin) are located primarily in the outer monolayer while the anionic zwitterionic lipids (phosphatidylethanolamine,

phosphatidylserine and phosphatidylinositol) are located primarily in the inner or cytoplasmic monolayer. In model systems the former lipids are usually more fluid while the latter lipids are more rigid¹⁰. Thus, a fluidity gradient could exist across the bilayer of biological membranes. Such a gradient and alterations of it may have important consequences in vectorial processes such as small molecule transport, receptor mobility, hormone receptor interactions and the activity of membrane-bound enzymes. However, as mammalian cells can compensate or homeoviscously adapt and because biological membranes are complex mixtures of cholesterol and phospholipids with different fatty acyl chain length or unsaturation, direct experimental measurement is necessary to determine if such vertical asymmetry of 'fluidity' exists across the plasma membrane bilayer of mammalian cells¹¹⁻¹⁶. I report here use of a fluorescent probe molecule, β -parinaric acid, used in conjunction with the amino labelling reagent 2,4,6-trinitrobenzenesulphonic acid (TNBS) to investigate this asymmetry. The data obtained are consistent with the conclusion that the inner monolayer of the plasma membrane is less fluid than the outer monolayer.

LM cells (ATCC-CCL 1.2), a strain of transformed murine fibroblasts, were grown in suspension in a chemically defined medium and plasma membranes isolated as described previously¹¹. The cells or isolated plasma membranes were labelled with TNBS under penetrating or nonpenetrating conditions as previously stated⁵. The computer-centred spectrofluorimeter of Holland *et al.*^{12,13} was used for all fluorescence measurements¹⁴⁻¹⁷.

As β -parinaric acid distributes rapidly across both sides of the plasma membrane, one method to determine the physical pro-

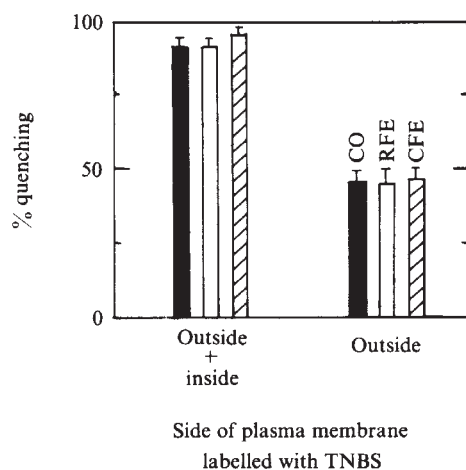


Fig. 1 Fluorescence quenching of β -parinaric acid in isolated LM cell plasma membranes by TNBS. β -parinarate quenching of outside+inside monolayers of the plasma membrane was determined as follows: plasma membranes were isolated as stated previously¹¹ and reacted under penetrating conditions with TNBS to trinitrophenyl-label all free amino-phospholipids in both monolayers of the plasma membrane as described elsewhere⁵. β -parinarate was incorporated into the plasma membranes at a β -parinarate/lipid molar ratio of 1:100 and absorption corrected fluorescence (CO), relative fluorescence efficiency (RFE), (CFE) and corrected fluorescence emission were determined at 313 nm, 313 nm and 415 nm respectively as described earlier¹⁴⁻¹⁷. The per cent quenching of β -parinarate was determined by comparison of the values for these fluorescence parameters with values for β -parinarate in equivalent samples that had not been reacted with TNBS. The degree of fluorescence quenching in the outside monolayer was determined by prelabelling the intact LM cells under nonpenetrating conditions with TNBS followed by membrane isolation and incorporation of β -parinarate into the membranes as above^{5,14-17}. Values indicate the mean $\pm$ s.e.m. ($n = 3$) and differences in each parameter are significantly different at $P < 0.01$ by Student's t test. Outside and inside refer to the side of the plasma membrane labelled with TNBS.

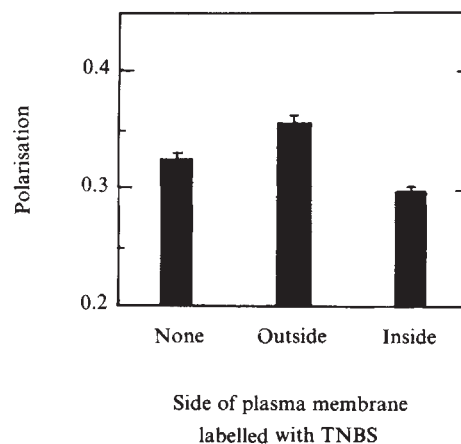


Fig. 2 Asymmetry of fluorescence polarisation between outer and inner monolayers of isolated LM cell plasma membranes. All methods were as described in the legend to Fig. 1 for [outside+inside monolayers] and [inside monolayer]. Polarisation values for the [outer monolayer] were calculated as described in the text. Polarisation was measured as described elsewhere. None, outside and inside refer to the side of the plasma membrane labelled with TNBS. Thus, polarisation values represent the side opposite to the side labelled with TNBS. Each value represents the mean $\pm$ s.e.m. ($n = 3$); all values are different at $P < 0.01$ by Student's t test.

perties of the inside monolayer of the plasma membrane would be to quench fluorescence of the probe in one monolayer and measure the fluorescence properties of the probe in the unquenched monolayer. The amino labelling reagent TNBS can be used as such a chemical quencher of β -parinarate fluorescence on one side of the bilayer membrane. Free TNBS has an absorption maximum in aqueous solution at 340 nm. However, after reacting with free amino groups a new absorption band near 420 nm appears^{18,19}. This absorption band overlaps with the lower wavelength side of the fluorescence emission spectrum of β -parinaric acid in plasma membranes and in blood lipoproteins and is therefore an ideal quenching agent if the quenching of β -parinarate fluorescence is nontrivial¹⁴⁻¹⁹. This quenching is nontrivial because the shape of the fluorescence emission curve of β -parinarate in the plasma membrane is unaltered by trinitrophenyl amino groups and the fluorescence of β -parinarate in organic solvents is quenched completely by trinitrophenylglycine, indicating high quenching efficiency of β -parinarate fluorescence by the trinitrophenyl group. If both sides of the plasma membrane were labelled by TNBS then fluorescence of β -parinarate should be quenched almost completely. When both sides of the isolated plasma membrane of LM cells are labelled with TNBS, absorption-corrected fluorescence, relative fluorescence efficiency and corrected fluorescence emission are quenched 91-95% (Fig. 1). Thus, most of the β -parinarate is accessible to quenching by energy transfer. If only the outer monolayer is labelled with TNBS, then these parameters are quenched only 44-46%. These results indicate that the trinitrophenylated amino groups of the outer monolayer cannot quench the fluorescence of β -parinarate in the inner monolayer. The data are also consistent with an approximately equal distribution of β -parinarate across both sides of the bilayer and indicate that the properties of the inner monolayer can be investigated independently of the outer monolayer.

The asymmetry of 'fluidity' across the plasma membrane bilayer is best demonstrated by the polarisation measurements shown in Fig. 2. Fluorescence polarisation is directly proportional to microviscosity. The polarisation of β -parinarate in plasma membranes that were not reacted with TNBS was 0.325 ± 0.005 while that in plasma membranes labelled on the outside with TNBS was 0.355 ± 0.006 . The polarisation of β -parinarate in the outer monolayer can be estimated to be 0.290, using the formula $P_{\text{inside+outside}} = xP_{\text{inside}} + (1-x)P_{\text{outside}}$ (where x

is the mole fraction of β -parinarate), assuming x to be 0.5. This seems to be a reasonable assumption in view of the results shown in Fig. 1. Thus, the minimum difference in polarisation between the inner and outer monolayer would be 0.030 (if almost all of the probes were located in the outer monolayer).

The data presented here indicate that the inner monolayer of plasma membranes from a tumorigenic cell grown in tissue culture may be more rigid (lower fluidity) than the outer monolayer. The function of such a fluidity gradient across the membrane bilayer is not known. However, it is known that both in normal and transformed cells the lipid composition of the membranes can be modified by medium constituents (see ref. 11 for review). Thus, alterations in or regulation of membrane fluidity gradients may be important in membrane pathologies. In support of this, several types of fluidity asymmetries have been suggested by electron spin resonance data. Red blood cell membranes appear to have a more rigid outer monolayer than inner monolayer, a finding opposite to that presented here²⁰. In contrast, sarcoplasmic reticulum membranes do not have an asymmetry of physical properties in the hydrocarbon region of the bilayer but an asymmetry similar to that presented here exists for the phospholipid polar head groups across the bilayer²¹. These results may point out basic differences in asymmetry of physical properties of membranes in mammalian cells.

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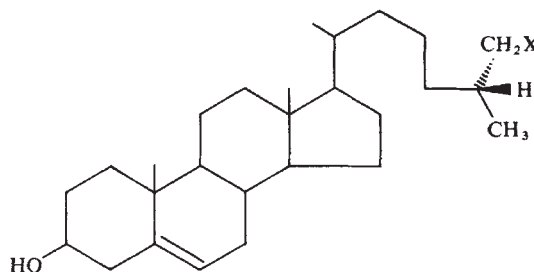


Fig. 1 The cholesterol analogue used to introduce a halogen atom into the bilayer. X is either bromine or iodine. The molecule is identical to cholesterol except for the exchange of a hydrogen atom on C-26 by the halogen. The (25*R*)-26-bromocholesterol, melting point 134–136 °C, NMR δ 3.32 (d, $J = 6$ c.p.s., $-\text{CH}_2\text{Br}$), was made by treating (25*R*)-26-hydroxycholesterol (prepared from kryptogenin) with *N*-bromosuccinamide and triphenylphosphine in tetrahydrofuran. Similarly, treatment of (25*R*)-26-hydroxycholesterol with *N*-iodosuccinamide and triphenylphosphine in tetrahydrofuran gave the (25*R*)-26-iodocholesterol, melting point 118–120 °C, NMR δ 3.2 (d, $J = 6$ c.p.s., $-\text{CH}_2\text{I}$).

derivation of such electron density profiles requires a knowledge of both the amplitudes of the X-ray reflections and their corresponding phase angles. Although the relative amplitudes can be easily determined from a diffraction pattern, the phases of the reflections cannot be directly observed. The determination of these phases has proved to be one of the most difficult and contentious aspects of the membrane diffraction field^{1–9}. In addition, because of the difficulty in measuring absolute intensities^{10,11}, the profiles are, almost invariably, calculated on a relative electron density scale. The lack of an absolute scale has often greatly hindered the interpretation of membrane profiles, particularly with regard to the distribution of the protein molecules^{9,12–16}. We describe here a novel method of directly determining both the phases of the X-ray reflections and an absolute electron density scale, and how the method has been successfully tested on lecithin/cholesterol bilayers.

The basis of the method is the isomorphous exchange of cholesterol in the membrane with a cholesterol analogue which is halogenated at C-26 (see Fig. 1). The introduction of this molecule into the membrane places an electron-dense atom close to the bilayer centre. Figure 2 shows schematically how the introduction of such a heavy atom affects the diffraction pattern for the simple case of a unit cell containing a single centrosymmetric lipid bilayer. Provided that the heavy atoms lie close enough to the bilayer centre, they will make only a positive contribution to the Fourier transform of the unit cell. When the halogen atoms are added, all reflections which have positive signs will increase in intensity, and the intensity of all those with negative signs will decrease. The phases of weak reflections, which may pass through zero on addition of the halogen atoms, can be checked by an experiment in which only half the original number of heavy atoms are introduced.¹⁷ Thus, all the phases can be directly inferred simply from the changes in intensity that occur when the halogen atoms are added to the bilayer.

If the observed electron density profiles for the control and heavy atom unit cells are on the same relative scale, then a multiplicative scale factor K will relate this scale to the absolute electron density scale. The value of K is easily obtained from the observed difference electron density profile, as the area under this profile is simply related to the total number of halogen electrons in the heavy atom unit cell. If the area under the difference profile, the mass density of the bilayer and the lamellar spacing are measured, and the contents of the unit cell are known, then K can be evaluated. However, the profile can still be translated with respect to the absolute scale. Its position on the scale can be fixed by knowing the absolute electron

A direct method for determination of membrane electron density profiles on an absolute scale

THE principal aim in X-ray diffraction studies of biological membranes and lipid bilayers is to determine the electron density profile across the membrane and to interpret this in terms of the distribution of the component molecules. The

density of any point on the profile (such as the water layer) or the mean electron density. An absolute scale for the bilayer is thus established.

This method of determining the phases of the X-ray reflections and an absolute electron density scale for the bilayer was tested on a lipid multilayer system composed of dimyristoyl lecithin and 40% (molar) cholesterol. High-resolution diffraction patterns were recorded from multilayers in which all, or a fraction of, the cholesterol molecules had been replaced by either 26-bromocholesterol or 26-iodocholesterol (see Fig. 1). All the diffraction patterns were qualitatively the same. They showed 12–13 orders of a lamellar spacing of about 50 Å and a broad, equatorially orientated band at 4.5 Å which showed the hydrocarbon region to be in a fluid state. When the cholesterol in the bilayers was replaced by halogenated cholesterol, the principal difference observed in the diffraction pattern was a change in the relative intensities of the lamellar peaks.

The introduction of either the bromine or iodine analogues did not affect the higher orders, but changed the intensities of the first seven orders; these were given signs according to whether their intensity had increased (positive sign) or decreased (negative sign). The phase assignments were verified by repeating the experiments with bilayers in which only half the cholesterol molecules had been exchanged with the cholesterol analogues. The signs for all the orders of the control data have

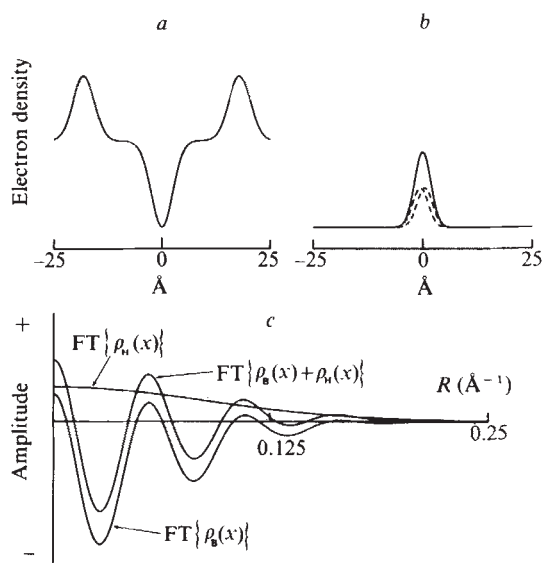


Fig. 2 An illustration of how the introduction of a heavy atom into the bilayer would change the diffraction pattern (see also ref. 8). The bilayer electron density profile $\rho_B(x)$ is shown in *a*. The Fourier transform of this distribution relative to the fluid layer outside the bilayer is labelled $FT\{\rho_B(x)\}$ in *c*. For a centrosymmetric structure this transform is real and has regions of positive and negative amplitude corresponding to phase angles of zero and π . The electron density distribution of the halogen atoms placed at distances $\pm X$ from the bilayer centre (in the figure $X = 1$ Å) and represented here by the sum of two Gaussians $\rho_H(x) = G[\exp(-(x+X)^2/\sigma^2) + \exp(-(x-X)^2/\sigma^2)]$, where G is the height and σ the characteristic width of each heavy atom gaussian, is shown in *b*. The Fourier transform of $\rho_H(x)$ is labelled $FT\{\rho_H(x)\}$ in *c* and is of the form

$$FT\{\rho_H(x)\} = F(R) = 2G\sqrt{\pi}\sigma \exp(-R^2\pi^2\sigma^2) \cos(2\pi RX)$$

where R is the reciprocal space coordinate. Thus, the addition of the halogen atoms to the bilayer results in a contribution to the unit cell Fourier transform which is a gaussian (which falls off slowly if the halogen atoms are well localised) modulated by a cosine term with a wavelength of $1/X$. The halogen atoms will therefore make a positive contribution to the unit cell transform up to a reciprocal space coordinate $R = 1/(4X)$, at which point the cosine first changes sign and the transform becomes negative. In the figure this occurs at $R = 0.25$ Å⁻¹.

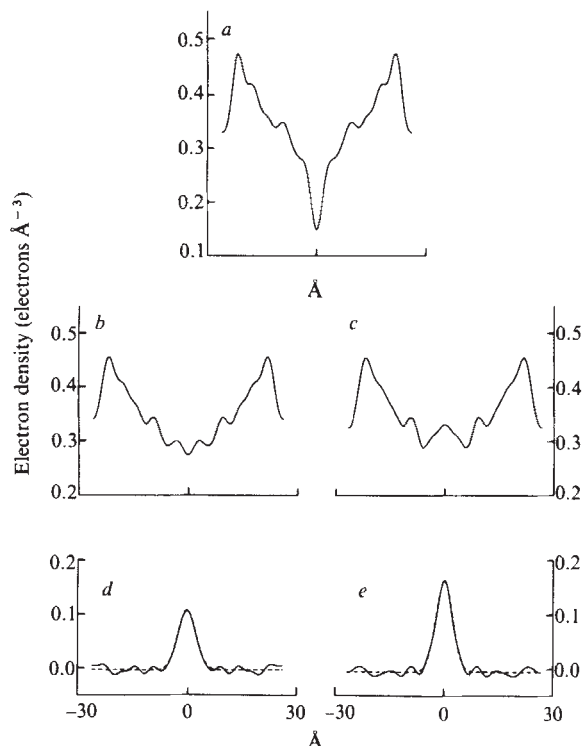


Fig. 3 Absolute electron density profiles of bilayers composed of dimyristoyl lecithin and 40% (molar) of *a*, cholesterol; *b*, 26-bromocholesterol; *c*, 26-iodocholesterol. The difference electron density profiles are shown in *d* and *e*. The profile of the bilayers containing 26-bromocholesterol is for a lamellar spacing of 52.12 Å and that of the bilayers containing 26-iodocholesterol is for a spacing of 53.20 Å. Profiles for bilayers containing normal cholesterol were calculated at both of these spacings using the swelling series data of N.P.F. and Lieb (ref. 18 and unpublished data). The determination of the absolute electron density scale is described in the text. The water contents of the multilayers were measured gravimetrically and the mass densities determined using an Anton-Parr precision density meter (we found $d_{\text{normal}} = 1.035$ g cm⁻³, $d_{\text{bromo}} = 1.074$ g cm⁻³ and $d_{\text{iodo}} = 1.090$ g cm⁻³). The cross-sectional areas of the unit cells (one lecithin plus 2/3 of a cholesterol) were calculated to be $A_{\text{normal}} = 67.31$ Å², $A_{\text{bromo}} = 68.25$ Å² and $A_{\text{iodo}} = 69.22$ Å². The mean electron densities for the bilayers were 0.345, 0.355 and 0.357 electrons Å⁻³, respectively, for the control, the bilayers containing 26-bromocholesterol and the bilayers containing 26-iodocholesterol. The assignment of phases for the first seven orders is described in the text; the phases for the higher orders were determined from swelling series and this work will be described elsewhere (N.P.F. and Lieb, in preparation). The samples were prepared on curved glass substrates from solutions of the lipids in 10:1 (v/v) chloroform/methanol as described previously²². The essential details of the experimental method and the correction of the diffracted intensities have been described previously⁶.

been independently determined by an extensive series of swelling experiments (N.P.F. and Lieb, unpublished) and are consistent with those determined here.

The electron density profiles for lecithin/cholesterol bilayers containing normal cholesterol, 26-bromocholesterol and 26-iodocholesterol are shown in Fig. 3*a-c*. Ignoring for the moment how the absolute electron density scales in Fig. 3 were determined, consider first the general features of the profiles. The control electron density distribution of Fig. 3*a* shows a familiar bilayer profile with peaks at about ± 22 Å from the bilayer centre, due to the phosphate groups of the lecithin molecules, and a well defined trough of low electron density in the centre of the bilayer where the terminal methyl groups are localised. The most striking change in the electron density profile that occurs when normal cholesterol is exchanged with either of the halogenated analogues is the large increase in electron density in the

centre of the hydrocarbon region (see Fig. 3*b,c*). This large change is accompanied by a small, but significant, smoothing of the fine detail in the profiles, interpreted as a small increase in disorder in the bilayer due to the introduction of the halogen atoms. Note that, apart from this slight overall increase in disorder, the effects of the halogen atoms are confined to a narrow region near the bilayer centre; the appearance of the head-group region and the distance across the bilayer between the phosphate groups remain unchanged.

Small increases in cross-sectional area were observed when the halogen atoms were added. This is consistent with the increased disorder observed in the one-dimensional electron density profiles. The scale factor between the control and heavy atom profiles was refined by minimising the difference in the profiles over the head-group region. The observed increase in disorder was accounted for in the refinement by applying a simple gaussian temperature factor¹⁹ to the control profile. Both this temperature factor and the scale factor were allowed to vary so as to minimise the difference.

The difference electron density profiles are shown in Fig. 3*d* and *e*. The electron density added to the bilayer by the introduction of the halogen atoms can be clearly seen to be confined to a region near the bilayer centre, the ripple in the background being within experimental error. The dashed line with each curve is the predicted electron density profile for a gaussian distribution of the appropriate halogen atoms placed at mean positions $\pm X$ from the bilayer centre. For both bromine and iodine, excellent agreement is obtained between the observed and predicted profiles when X is 0.8 Å and 0.2 Å, respectively, with a Debye–Waller temperature factor of 420 Å².

In order to put the profiles of Fig. 3 on an absolute electron density scale, the multiplicative factor K was first evaluated. It was found that the scale factor for the two halogens agreed to within 4%, thus giving a measure of the overall precision of the absolute scale determination. The mean of the two values was used for the scale shown in Fig. 3. Having determined the multiplicative scale factor the profiles could be fixed on the scale by using the calculated values for the mean electron densities of the bilayers. Where comparison can be made (for example, for the lecithin head-group region) the electron density scale derived here agrees well with values deduced from partial specific volume data and crystallography^{20,21}. The absolute scale imposes important constraints on molecular models of these bilayers. We hope that this new method of determining phases and an absolute electron density scale will be extended and applied to both natural membranes and to protein-containing model systems.

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Role of DNA polymerase γ in adenovirus DNA replication

THE linear DNA of the human adenoviruses type 2 and type 5 (Ad2, Ad5) replicates in the nucleus of permissive cells by a mechanism different from that of chromosomal DNA or papovavirus DNA. Papovaviruses replicate bidirectionally, with both parental strands being duplicated almost synchronously, whereas adenovirus DNA replication occurs unidirectionally by a strand displacement mechanism¹. No new DNA polymerase has been observed in Ad5 infected cells². To determine which cellular DNA polymerase is required for adenovirus DNA replication we used the nucleotide analogue 2',3'-dideoxythymidine triphosphate (ddTTP) which inhibits DNA polymerase β , and in particular DNA polymerase γ but not DNA polymerase α (refs 3, 4). Addition of ddTTP to isolated nuclei which synthesise adenovirus DNA *in vitro* results in a strong inhibition. In similar conditions cellular DNA or SV40 DNA synthesis is not affected. In view of the presence of DNA polymerase α and γ , but not β , in adenovirus replication complexes^{5,6} our results indicate that only DNA polymerase γ is required for adenovirus DNA chain growth.

KB cells were infected with Ad5 or Ad2 and 16 h after infection, when viral DNA replication is maximal, nuclei were isolated. The nuclei were incubated at 32 °C in the presence of the ingredients necessary for *in vitro* DNA synthesis⁷ and various concentrations of ddTTP. ³H-dGTP was used as the radioactive label and dATP, dCTP and dTTP were present at 50 μ M. After 30 min the reaction was stopped, viral DNA was selectively extracted and ³H-dGMP incorporation was measured after precipitation by trichloroacetic acid. The results (Fig. 1) show that adenovirus DNA synthesis is very sensitive to ddTTP, with 50% inhibition at a ratio (ddTTP)/(dTTP) = 1.4×10^{-2} . Cellular DNA synthesis is not inhibited up to a ratio (ddTTP)/(dTTP) = 20. When ddATP is used as nucleotide analogue, instead of ddTTP, similar results are obtained (data not shown).

We have also studied the effect of ddTTP in nuclei from primary monkey cells (AGMK). These cells are permissive for SV40 but are semi-permissive to adenovirus infection, caused by a block in some late event without affecting viral DNA synthesis^{8,9}. Hence, AGMK cells provide the possibility to study the effect of ddTTP on two different viral systems in one type of host cell. Figure 1 shows that also in AGMK cells Ad5 DNA synthesis is very sensitive to ddTTP while SV40 DNA synthesis is only inhibited when ddTTP is present in at least 20-fold excess compared to dTTP. This confirms the results of Edenberg *et al.*³ for SV40 DNA synthesis in nuclear lysates.

The inhibition of adenovirus DNA synthesis *in vitro* is independent of the time of incubation and can be suppressed by an excess of dTTP. At 500 μM dTTP, 1 μM ddTTP inhibited only 4% while at 50 μM dTTP 60% inhibition was observed, with the same concentration of ddTTP. Evidence for the competitive character of ddTTP inhibition was obtained by varying the nucleoside triphosphate concentration (S) in the presence of different ddTTP concentrations. Straight parallel lines were observed when S was plotted against S/V (not shown), giving an apparent K_m of 1.1 μM for dNTP and a K_i of 0.08 μM for ddTTP.

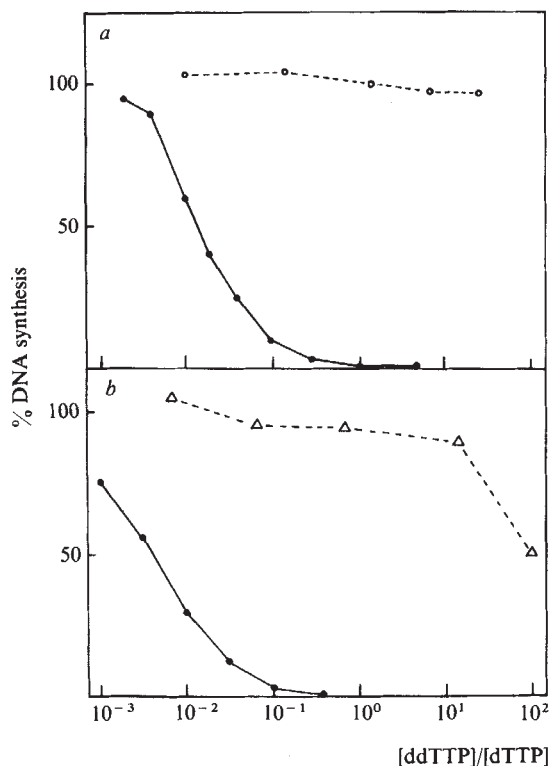


Fig. 1 Inhibition of adenovirus DNA synthesis in isolated nuclei by ddTTP. KB cells (a) or AGMK cells (b) were infected with Ad5 (○) or SV40 (△). At 16 h (Ad5) or 40 h after infection (SV40) the cells were collected and nuclei were prepared⁷. The nuclei were incubated in a final volume of 0.1 ml (2×10^7 nuclei per ml) in a reaction mixture for DNA synthesis, containing ddTTP (PL Biochemicals) as indicated. Final concentrations were 40 mM *N*-2-hydroxyethylpiperazine *N'*-2-ethane sulphonate (pH 7.8), 55 mM NaCl, 45 mM KCl, 5 mM MgCl_2 , 0.4 mM CaCl_2 , 1 mM dithiothreitol, 1 mM phosphoenol pyruvate, 2 mM ethylene-glycol tetra-acetate, 2 mM ATP, 0.05 mM each UTP, CTP, GTP, dATP, dCTP, dTTP, and 1 U ml^{-1} pyruvate kinase. ^3H -dGTP was present at 5 μM and 2 Ci mmol^{-1} . After 30 min at 32 °C the reaction was stopped by addition of 1 ml of 0.01 M Tris-HCl (pH 7.5), 0.01 M EDTA and cooling. To uninfected or Ad5-infected nuclei 1 ml 10% TCA containing 0.01 M sodium pyrophosphate was added. After 15 min TCA-precipitable material was collected on Whatman GF-C filters, washed with 50 ml 0.5% TCA-1 mM sodium pyrophosphate and with 96% ethanol, dried and counted in a liquid scintillation counter. From part of the nuclei, before addition of TCA, Ad5 DNA was selectively extracted⁷ and the viral nature of the newly synthesised DNA was established by CsCl gradient centrifugation. SV40 DNA synthesis was determined by extraction of the DNA¹⁷ followed by TCA precipitation. Part of the SV40 DNA was analysed by sucrose gradient velocity sedimentation in the presence of ^{32}P -labelled marker DNA. The DNA sedimented at 22–28 S with a pattern characteristic for SV40 DNA synthesised *in vitro*¹⁸. 100% = 20,530 c.p.m. per 10^6 nuclei (Ad5), 2,530 c.p.m. per 10^6 nuclei (uninfected) and 7,241 c.p.m. per 10^6 nuclei (SV40). ○, Uninfected KB cells.

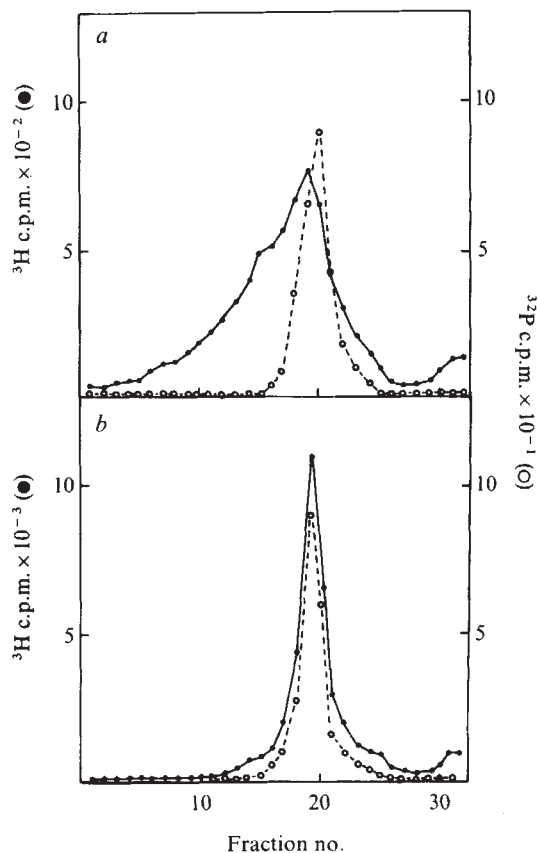


Fig. 2 Neutral sucrose gradient sedimentation of adenovirus DNA synthesised in isolated nuclei in the presence (a) or absence (b) of ddTTP. KB cells were infected with Ad5 and 16 h after infection nuclei were isolated. The nuclei were incubated as described in Fig. 1 in the presence of 2 μM ddTTP ((ddTTP)/(dTTP) = 4×10^{-2}) which gives 80% inhibition compared to the control, without ddTTP. Viral DNA was extracted⁷ and dialysed against 0.01 M Tris-HCl pH 8.0–0.01 M EDTA–1 M NaCl. Samples obtained from 2×10^6 nuclei were mixed with ^{32}P -labelled Ad5 DNA and layered on a 5–27% isokinetic sucrose gradient in 0.01 M Tris-HCl pH 8.0–0.01 M EDTA–1 M NaCl–0.1% Sarkosyl. Centrifugation was performed for 16 h at 24,000 r.p.m. at 4 °C in a SW 41 rotor. Sedimentation is from right to left. When tested in a CsCl equilibrium centrifugation run all ^3H -labelled DNA had the expected density of Ad5 DNA, 1.761 g cm^{-3} .

To study the mechanism of inhibition, Ad5 DNA synthesised in the presence of 2 μM ddTTP, which gives 80% inhibition, was analysed by neutral sucrose gradient centrifugation (Fig. 2). 67% of the newly synthesised DNA sedimented between 31S and 68S, which is the sedimentation range of replicative intermediates, while in the control, without ddTTP, only 7% replicating DNA was found. In alkaline conditions, the viral DNA made in the presence of ddTTP appeared as molecules smaller than genome length (data not shown). These results can be explained by assuming that ddTTP is incorporated into growing DNA chains and acts as a chain-terminator, a mechanism that is proposed for the action of ddTTP on DNA polymerase I of *Escherichia coli*¹⁰ and for the effect of ddTTP on ΦX174 DNA replication in permeabilised *E. coli*¹¹.

DNA polymerases α , β and γ from CV1 cells or HeLa cells can be distinguished by their different sensitivity to ddTTP^{3,4}. We have confirmed these results with purified KB-DNA polymerases (Fig. 3). DNA polymerase α is most resistant with 50% inhibition at ((ddTTP)/(dTTP) = 30. DNA polymerase β is partially resistant (50% inhibition at ((ddTTP)/(dTTP) = 1) while DNA polymerase γ is most sensitive. When assayed with

activated DNA in the presence of 5 mM Mg^{2+} , 50% inhibition was found at $(ddTTP)/(dTTP) = 6 \times 10^{-2}$ while in the presence of poly (rA)-oligo(dT) as template-primer and 0.5 mM Mn^{2+} , the preferred test conditions for DNA polymerase γ , the enzyme was inhibited even more markedly by the nucleoside analogue. A similar result was obtained with ddATP as inhibitor in the presence of activated DNA.

In view of the large difference in sensitivity between DNA polymerases α and γ and the specific inhibition of adenovirus DNA synthesis by ddTTP and ddATP, our results can be best explained by assuming that DNA polymerase γ is required for chain elongation of adenovirus replicative intermediates. The concentrations of ddTTP giving 50% inhibition of both adenovirus DNA synthesis and DNA polymerase γ activity are of the same order of magnitude and ddTTP behaves as a competitive inhibitor in both situations. Caution is required, however, when extending results obtained with purified enzymes to the more complicated situation that exists in the replication fork. A function of DNA polymerase β in viral DNA chain growth is less likely since only α and γ enzymes are found in replication complexes capable of elongation^{5,6} and since adenovirus DNA synthesis is sensitive to low concentrations of *N*-ethylmaleimide, which do not inhibit DNA polymerase β activity.

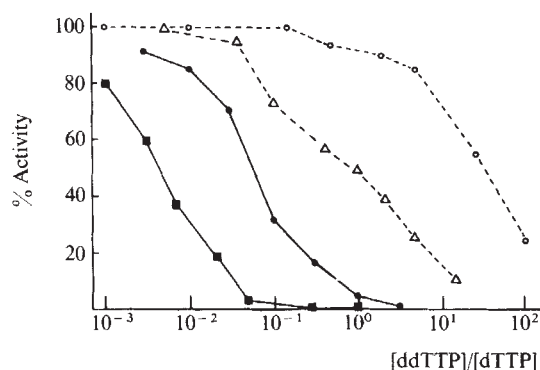


Fig. 3 Effect of ddTTP on DNA polymerase α , β and γ . DNA polymerases α and β from KB cells were purified by DEAE cellulose and DNA cellulose chromatography². DNA polymerase α was characterised by sedimentation (8.8S) and its sensitivity to *N*-ethylmaleimide (95% inhibition by 1 mM NEM). DNA polymerase β sedimented at 3.4S and was completely resistant to 5 mM NEM. DNA polymerase γ was isolated according to Knopf *et al.*¹⁹ and purified by DEAE-cellulose, phosphocellulose, hydroxylapatite and DNA cellulose chromatography. The enzyme preferred poly (rA)-oligo(dT) as template-primer to activated DNA, was 85% inhibited by 5 mM NEM and was essentially free of contaminating DNA polymerase α or β , as indicated by the absence of remaining enzyme activity at a $(ddTTP)/(dTTP)$ ratio >1 . The DNA polymerases were tested¹⁹ with 5 mM NEM present in the β -polymerase incubation mixture. $\circ$, DNA polymerase α ; $\triangle$, DNA polymerase β ; $\blacksquare$, DNA polymerase γ with poly (rA)-dT₁₂₋₁₈ as primer-template; $\bullet$, DNA polymerase γ with activated DNA as primer-template.

The function of DNA polymerase γ in the cell is unknown. Recently, several laboratories¹²⁻¹⁴ have described the identical properties of nuclear DNA polymerase γ and mitochondrial DNA polymerase. Interestingly, both mitochondrial DNA¹⁵ and adenovirus DNA replicate unidirectionally according to a strand-displacement mechanism, in contrast to nuclear DNA synthesis or papovavirus DNA replication. Another significant difference is the absence of histones in intracellular adenovirus DNA¹⁶. The above differences, together with our results, indicate the presence of at least two enzymatically distinguishable replication mechanisms in the mammalian cell.

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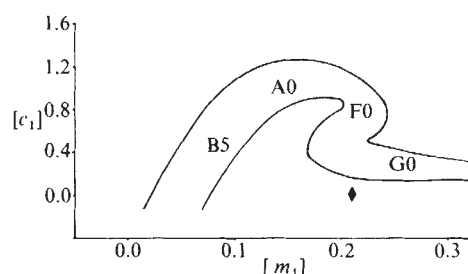
Errata

In the article 'Nucleotide sequence of bacteriophage G4 DNA' by G. N. Godson, B. G. Barrell, R. Staden and J. C. Fiddes, *Nature* **276**, 236-247, the two sentences starting on line 2 of page 242 should read: 'The amino acid differences between the proteins of the two phages is shown in Table 1. Gene *D* is the most highly conserved protein between the two phages, with only 17.8% of the amino acids changed, but this may be partly due to the presence of the overlapping *E* gene within the gene *D* coding region (see below).'

In the letter 'Are receptor-activated ciliary motor responses mediated through voltage or current?' by J. de Peyer and H. Machemer, *Nature* **276**, page 285, the last sentence in paragraph 1 should read: 'This is the first report of intracellularly recorded receptor currents in ciliates.'

In the letter 'Site of 1,25(OH)₂-vitamin D₃ synthesis in the kidney' by M. G. Brunette *et al.*, *Nature* **276**, page 287, line 16 in paragraph 3 should read: 'were less readily digested by collagenase, and could not be dissected'

In the letter 'An ultraviolet subdwarf companion to HD17576' by J. Darius and P. A. Whitelock, *Nature* **275**, page 428, Fig. 2 was printed without the diamond symbol representing the position of HD17576. The figure is shown correctly below.



matters arising

Large circles on the Earth's surface

AT the 3rd International Conference on Basement Tectonics in Durango on 15–19 May 1978, slides of the large circles in Arizona described by Saul¹ were shown. Several viewers pointed out that the largest circle in Fig. 1 of this paper had also been seen on satellite (Landsat) images, contrary to Saul's experience, and that such images are advantageously used in conjunction with the photographs of raised relief maps.

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1. Saul, J. M. *Nature* **271**, 345–349 (1978).

SAUL REPLIES—Several investigators have noted that some of the strongest circles are visible on satellite photographs and that some are also discernible on geological and tectonic maps. Satellite photographs of areas that have partial snow cover seem particularly useful. At present the original photomaps made by James Lindsay seem to be the most informative. Lindsay had noted the largest circle in Fig. 1 ref. 1 several years ago as well as strong linear features, some of which have apparently not been noted on more conventional types of coverage. The appearance of these linear features is strongly dependent on the direction of lighting. Several people pointed out circular features in the Canadian Shield, South Africa, East Africa, South Australia and the Himalayan region but these were found on a great variety of maps, photographs and plots, and their identity with those produced by photographing raised relief maps was surmised rather than demonstrated. One of the most interesting was the circular pattern noted by G. A. Kellaway for tin lodes in Cornwall. S. Parker Gay, Jr has identified large circles using the techniques he has developed for viewing geophysical data and topographic information in stereoscopic pairs².

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2. Gay, S. P., Jr *Geophysics* **36**, 396–414 (1971).

Lifetime of an elliptical ring around Uranus

LUCKE¹ suggests that Uranus' ϵ ring is composed of particles in elliptical orbits, with slightly different eccentricities and nearly perfect alignment of orbital axes. This model can fit observational data^{2–4} obtained in 1977 during the occultation of the star SAO158687. We point out that the model probably works only if the ring is a short-lived configuration, because perturbations due to Uranus' oblateness quickly change the shape of the ring.

If we consider the precession period P of apsidal lines in a ring lying in the planetary equatorial plane, we have

$$P = \frac{4\pi a^{7/2}(1-e^2)^2}{3J_2 R^2 (GM)^{1/2}} \approx 98 \text{ d} \quad (1)$$

where M , R , J_2 are the mass, radius and form factor of Uranus respectively (this latter is not well known; we use a typical value of 0.01 (ref. 5)); a and e are the semimajor axis and eccentricity of the satellite particle. If two particles in the ϵ ring have semimajor axes and eccentricities differing by Δa and Δe , the precession periods differ by a quantity

$$\Delta P = \left(\frac{7\Delta a}{2a} - 4 \frac{e\Delta e}{(1-e^2)} \right) P \quad (2)$$

and a randomisation of axis direction is likely to occur in a time

$$T \approx P^2 / \Delta P = P / \left(\frac{7\Delta a}{2a} - 4 \frac{e\Delta e}{1-e^2} \right) \quad (3)$$

It is unlikely that all pairs of particles have orbits with a and e differing by just the quantity needed to make the denominator of equation (3) very small. Therefore, an upper limit on T may be obtained by assuming $\Delta a = 0$, $e = 0.008$ and $\Delta e = 0.0005$ (the latter values are chosen to account for the width variation of the ring¹). We get in this case $T \approx 6.3 \times 10^4 P \approx 16,900 \text{ yr}$. A more reliable value of T may be obtained if we consider that interparticle collisions are not likely to allow $\Delta a = 0$; for $\Delta a/a \approx 10^{-3}$ (which corresponds to the width of the ring), we get $T \approx 300 P \approx 80 \text{ yr}$.

A similar phenomenon occurs if the ring lies out of the equatorial plane. In this case, the precession of the line of nodes yields, in a time of the order of T , a symmetrical band (with respect to the equatorial plane) that subsequently

flattens out as a result of inelastic collisions among particles⁶.

We conclude that the ϵ ring, if composed of particles in elliptical (or inclined) orbits around Uranus, may be only a transient phenomenon, and that the ring perhaps evolves in a way which may be observed.

We thank G. Colombo for useful discussions.

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Matters Arising

Matters Arising is meant as a vehicle for comment and discussion about papers that appear in *Nature*. The originator of a Matters Arising contribution should initially send his manuscript to the author of the original paper and both parties should, wherever possible, agree on what is to be submitted. Neither contribution nor reply (if one is necessary) should be longer than 300 words, and the briefest of replies, to the effect that a point is taken, should be considered.

Why should the genome congeal?

A NUMBER of theoretical studies during the past decade have dealt with the problem "Why does the genome not congeal?"¹ that is, why are co-adapted gene combinations repeatedly reshuffled? As a result, specific conditions which may favour recombination have been de-

ried²⁻⁸. However, such conditions are rare and pertain only to restricted segments of the genome. Therefore, selection favouring recombination should be the exception rather than the rule and hence Maynard Smith's conclusion "such models explain why the genome does not congeal, but not why recombination is as free as it is"⁹. This discrepancy between the observed and expected recombination frequencies may stem from an oversimplification inherent in the theoretical approach; namely, the consideration that recombination within a given chromosomal region is independent of other regions. This assumption may be unfounded in view of increasing data clearly indicating the interrelation of recombination frequencies within different chromosomal regions.

A recombination control system¹⁰ involves controlling elements (genes determining recombination frequencies) and controlled elements (chromosomal sites recognised and acted upon by the products of specific controlling genes). The recognition sites of any recombination control system are not necessarily linked to the controlling genes, but may be distributed over the entire genome. Consequently several unlinked regions may be controlled by the same gene, as demonstrated in *Schizophyllum*¹¹⁻¹³ and *Neurospora*^{14,15}. On the other hand, a chromosomal segment flanked by any two recombining markers, A and B, may well include recognition sites pertaining to different control systems. Thus, recombination rates between two particular markers may be influenced by several different controlling genes, a situation known to exist in *Schizophyllum*^{16,17}, *Tribolium*¹⁸ and *Neurospora*¹⁴.

If the region between A and B includes recognition sites for the controlling loci C_1 , C_2 and C_3 , recombination between A and B will be influenced by the particular alleles at C_1 , C_2 and C_3 . Selection favouring recombination between A and B may lead to an increase in the allelic frequencies of high recombination alleles at all three loci. As these loci control recombination rates in additional regions as well, any increase in high recombination alleles may elevate recombination rates in regions other than A-B. Consequently, selection favouring recombination within a particular chromosomal region may result in increased recombination rates elsewhere. Therefore, high recombination frequencies between two given markers may occur as an indirect result of selection favouring recombination in another region. Theoretically, the same indirect effect should also be apparent when recombination within a given region is disadvantageous. However, since low recombination rates are directly selected for over most of the genome, the indirect effect of selection against recombination is expected to be less obvious.

Practically all investigations of naturally occurring recombination-control genes conclude that low recombination is dominant to high (as shown in *Neurospora*¹⁵, *Tribolium*¹⁸, *Ustilago*¹⁹, *Drosophila*²⁰ and *Schizophyllum*²¹). These experimental results concur with the theoretical model⁶ predicting that individual selection for recombination would favour recessive high recombination alleles.

Dominance relationships between controlling alleles are particularly relevant to two aspects of selection affecting recombination rates. First selection against a recessive trait. Selection against recombination is essentially selection against a recessive trait^{15,18-21}, a process rarely culminating in fixation. Therefore, high recombination alleles would remain in the gene pool despite vigorous and long-term selection against recombination. Second, the 'hitchhiking effect': Strobeck *et al.*⁵, proposing hitchhiking as the mechanism for the propagation of high recombination genes, had to assume close linkage between the controlling gene(s) and the recombining markers. This assumption considerably weakens the efficiency of selection favouring recombination. However, if high recombination is indeed recessive, this assumption is unnecessary. In that case, an individual producing recombinant gametes must be homozygous at the controlling locus (loci) and the recombinant gamete will necessarily carry the high recombination allele(s). Consequently, selection favouring recombinants will result in the increase of high recombination allele frequency, regardless of its linkage relationship with the recombining markers.

Selection affecting recombination rates is operant on various levels. The rate of recombination between A and B may be altered by allele substitution at any of the relevant controlling loci. Alternatively, recombination frequency may be affected by changes in recognition sites lying between loci A and B. Although the nature of these recognition sites is still unknown, there is evidence that genetic control of recombination requires specific combinations of nucleotide sequences on the two homologues involved²². A particular sequence on one chromosome only is insufficient, thereby simulating a relationship paralleling, though not identical to, recessivity. Therefore, the situation concerning recognition sites may be similar to that of controlling genes, as far as the efficiency of selection against them is concerned.

The genetic control of recombination has evolved into a system that practically ensures the almost infinite storage of a high potential of recombination. In such a system, no population can effectively eliminate the controlling and controlled elements for high recombination. Consequently, even though selection favouring recombination is weak, tran-

sient and localised, it may well balance the persistent selection pressure against recombination.

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MAYNARD SMITH REPLIES—Dr Schaap makes two points. First, as loci affecting recombination do so in more than one region, higher recombination in any one region can be increased by selection, not only on the region itself, but at many others. Hence, she suggests, the total selection for higher recombination may be stronger than one would think from simple models. This is true enough. The snag is that the same is true of selection for lower recombination. I do not understand her argument that "since low recombination rates are directly selected for over most of the genome, the indirect effect of selection against recombination is expected to be less obvious".

Her second point, that alleles for higher recombination are usually recessive, is an interesting one, because selection for higher recombination arising from 'hitchhiking' is more effective in these circumstances. This tends to confirm my conviction that hitchhiking effects are the main ones responsible for maintaining high recombination.

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reviews

Founders of X-ray analysis

M. F. Perutz

William Henry Bragg, 1862–1942: Man and Scientist. By G. M. Caroe. (Cambridge University Press: Cambridge and London, 1978.) £8.95.

W. H. BRAGG became famous for his early work on alpha rays, for founding X-ray crystallography together with his son W. L. Bragg, and as a superb expositor of science. This book by his daughter is an affectionate memoir, vividly written, and illustrated with pictures and quotations. It has some of the lightness of touch of Gwen Raverat's *Period Piece* which conjured up the lives of the Darwins. I only just knew W. H. Bragg as a benign and dignified looking old gentleman, but I worked with his son W. L. Bragg while he was Cavendish Professor of Physics at Cambridge from 1939 to 1953 and remained associated with him until his death in 1971. Mrs Caroe's sensitive portrait told me much about her father that also illuminates the son. The book will interest many people: physicists and crystallographers, historians of science, those concerned with the social role of science, with science education, with the relationships between science and government, and between science and religion.

W. H. Bragg was the son of a merchant seaman turned farmer in West Cumberland. He won an exhibition to Trinity College, Cambridge, read mathematics and finished 3rd wrangler. One day in 1885 he walked along King's Parade with J. J. Thomson, another Trinity man with whom he often played tennis. Thomson asked whether the senior wrangler had applied for the chair in mathematics and physics at Adelaide. Bragg said, "No" and wondered if he himself might stand a chance. Thomson encouraged him to apply. The appointments committee consisted of J. J., of Horace Lamb, the mathematician who had just left the chair, and of the Agent-General for South Australia. The only other candidate could not be trusted with the bottle, and so they settled for the 23-year-old Bragg who abstained from drink. Happy days!

And more to come. Young Bragg had been brought up to work and no play, and had been poor, shy and lonely at Cambridge. He blossomed out in sunny Adelaide where he enjoyed the princely salary of £800 a year and

did not feel his humble origins as a social handicap. He threw his energy into work for the new University and community, married the daughter of the Government Astronomer, and became much liked and respected. The Australian setting with its pioneering enthusiasm, its outings and easygoing social life is charmingly described, as when Mrs Caroe tells us that her grandmother would try to keep her grandfather at home before parties lest he invited everyone he met in the street.

It never occurred to Bragg to do any research until, aged 42, he had to address the Australian Association for the Advancement of Science. He chose the ionisation of gases. In preparing his lecture he came across a puzzling observation by Madame Curie which showed that alpha rays were not subject to an exponential absorption law, like other radiations, but penetrated a certain distance and then suddenly stopped, like bullets shot into a piece of wood. Bragg argued that they could not be colliding with other atoms but must be passing right through them, losing energy only by the ionisation they caused on the way.

Bragg procured some radium, built an ionisation chamber and soon proved his point. He sent his first paper to Rutherford at McGill for comment and waited an interminable 3 months for a reply. When Rutherford congratulated him:

"I think you have struck the right explanation of the absorption of alpha rays and that it is merely a matter of careful experiments along the lines you are following to disentangle it completely."

Bragg replied:

"It is very jolly to have your comments on my paper."

This was the start of a regular and very interesting correspondence, much of which is quoted. Bragg next discovered that his preparation shot out alpha particles of four different ranges, corresponding to the four different active substances that Rutherford had shown to exist, and that the particles of shortest range came from radium itself.

Bragg then turned to gamma rays, and Mrs Caroe describes how their exploration and that of the related X-rays sent him off on a wrong tack. How could cathode rays impinging upon a target produce X-rays and these same X-rays, on hitting another target, produce secondary cathode rays? If

they were electromagnetic pulses, as Stokes had suggested, then why were these secondary electrons shot out mainly in the forward direction instead of parallel to the transversely vibrating field? And why were gamma rays and X-rays themselves scattered most in the forward direction instead of uniformly in all directions?

In 1907 Bragg wrote to Rutherford: "I have got the decisive experimental proof that gamma rays are not pulses, but corpuscular."

In a later lecture he said:

"The Röntgen ray must move in a straight line without loss by the way, so that when it strikes the fatal atom it may have an undiminished amount of energy to give to the electron which takes its place, and the velocity of the latter may not depend on the distance the ray has travelled from the tube . . . We must replace our spreading waves by clouds of minute bundles of energy, tiny entities which move like material particles, but with the speed of light. It is a curious return in the direction of the old Newtonian corpuscular hypothesis."

He suggests that the electron, as it is driven against the anticathode, picks up a neutralising amount of positive charge, producing a neutral pair incapable of deflection by fields.

"The neutralising positive may be torn away again from the electron in some other transit across an atom, and the Röntgen ray became once more a cathode particle."

Rutherford was enthusiastic at first, but later wrote more cautiously:

"I quite appreciate the utility of the physical conception in your doublet, but I would at any time be prepared to consider it a bundle of concentrated energy which has all the properties of your doublet."

Some of Bragg's difficulties would have been resolved if he had studied Einstein's 1905 paper on the photoelectric effect, but in a letter to Rutherford at the end of 1911 Bragg admits that he had not heard of Einstein when he formulated his theory. In another letter he comforts himself with what must have been a garbled message:

"Lindemann says that Planck's theory now is that the atoms can take in energy of any wavelength at any rate, but can only give it out in definite amounts—relativity is rather in the background in Berlin just now."

I found this account fascinating.

In 1909 Bragg's half-dozen papers on ionising radiations brought him an FRS and the physics chair at Leeds. Happy days! the reader may think again, but in fact it was the end of the happy days. While students and public at Adelaide

had lapped up Bragg's vivid lectures, they fell flat among the sober Yorkshiremen. Mrs Bragg could not get used to the dirt, the smoky dark, the poverty and the rickety children of Leeds, and Bragg's neutral pair theory ran into stormy seas. Yet amidst the acrimonious controversy with Barcla his greatness shows. He does not withdraw into cynicism, but writes to Rutherford:

"Supposing the identity of X-rays and light to be established, the supposition is this, I take it: The energy travels from point to point like a corpuscle: the disposition of the lines of travel is governed by wave theory. Seems pretty hard to explain; but that is surely, how it stands at present." Mrs Caroe's quotations from Bragg's early papers and lectures already show his talent for expressing science in lucid, homely language free from jargon.

Meanwhile Bragg's son, William Lawrence, having graduated in mathematics at Adelaide at 18, became an undergraduate once more at Cambridge, reading mathematics and physics. He writes:

"Dear Dad, I'm so glad you liked the notes on Jeans. I got an awful lot from a Dane who had seen me asking Jeans questions. He was awfully sound, and most interesting, his name was Böhr or something that sounds like it."

At Leeds in the summer holidays of 1912 he found his father intensely excited about the paper by von Laue, Friedrich and Knipping on the diffraction of X-rays by crystals. Back in Cambridge, he writes to his father:

"I want to have a good go at this crystal divining . . . But don't let on for a bit till I can try the crystal Pope (the Professor of Chemistry) is getting for me." Later:

"I have just got a lovely series of reflections of the rays in mica plates with only a few minutes' exposure! Huge joy." And the father writes:

"My dear Rutherford, my boy has been getting beautiful X-ray reflections from mica sheet just as simple as the reflections of light in a mirror,"

but in Cambridge the son was teased for having disproved his father's corpuscular theory of X-rays. The discovery of Bragg's law, the interpretation of the Germans' diffraction patterns and the determination of the structure of common salt were all done by the son alone at Cambridge.

In Leeds meanwhile the father put his experience with ionisation chambers to good use in constructing an X-ray spectrometer, with which father and son solved the structure of diamond and other simple crystals. Together they founded the science of X-ray analysis; the world reacted thoughtlessly, as though the decisive advance had been made by the father, helped along by the son. The father made matters worse by referring in a literally patronising way, to his "boy's" work, when the "boy" was craving for recognition as an independent scientist.

So their great discoveries, which brought them the Nobel Prize for Physics in 1915, tragically strained relationships between them for the rest of their lives. In his many lectures on the development of X-ray analysis W. L. Bragg was fond of defining the exact roles played by himself and his father, but he never hinted at those strains until a few days before his death when he wrote to me:

"I hope that there are many things your son is tremendously good at which you can't do at all, because that is the best foundation for a father-son relationship"



W. L. Bragg with his father, shortly before the latter's death in 1942

—a reflection on the painful competition between himself and his father. Mrs Caroe alludes to the conflicts between them with affection and sympathy for both.

In 1923 Bragg father became Director of the Royal Institution where he remained until his death 19 years later. Here he founded a flourishing school of X-ray crystallography from which much of today's biological structure analysis is descended. He also found scope for his superb gifts of popularising science, especially to children, and became a protagonist of the role that science ought to play in education, government and industry. He wanted children to learn more science, but opposed a purely scientific education that turns out backroom boys.

"If research workers' education were made more general, they would move into the Councils of industry to which specialists in science are as yet rarely admitted. The scientific expert must himself take down the barricade that makes the alley blind."

To this end he admonished scientists to make themselves understood:

"We cannot compel men to make use of science in the right way, but the chance that good use will be made is in a curious way dependent on the ease with which it is stated."

Unlike Monod in *Chance and Necessity*, who tries to base a new ethics on scientific truth, Bragg thought science was neutral:

"Science is merely knowledge. It has no morality at all. The achievements of scientists have value of one kind, and the spirit in which they worked has value of another kind. Faraday's reverence for truth and unselfish devotion to its acquisition have a higher value than the laws which he established."

Perhaps this is what Monod meant. Bragg regarded science as an ever receding frontier.

"There is a hesitation which would beg us not to push forward lest we come to think less of the world. As against this, research is an act of faith in the immensity of things. There is no end to the search: it is a poor thought that there might be." "The work of discovery will go on and no one can stop it."

Who would dare to say this today?

In his last year at school Bragg went through a harrowing religious experience, reminiscent of the one described so movingly in James Joyce's *Portrait of the Artist as a Young Man*. A less level-headed person might have reacted by turning against religion, but Bragg tried to bring religion into harmony with science, because he had faith in both,

"From religion comes a man's purpose; from science his power to achieve it. Sometimes people ask if science and religion are not opposed to one another. They are: in the sense that the thumb and fingers of my hand are opposed to one another. It is an opposition by means of which anything can be grasped."

His daughter writes of him:

"Religious faith to him was the willingness to stake his all on the hypothesis that Christ was right and test it by a lifetime's experiment in charity."

His charity found expression in the help he gave to Jewish scientists when they were driven from Europe by the Nazis, and again when they were misguidedly interned here as potential spies during the Second World War. In general, Bragg spoke only of the benefits that science would bring, but once he wrote:

"If it falls into the hands of evil persons working for evil ends we, as scientists, can do nothing about it."

I am surprised that he should have taken that easy line, perhaps because he was spared the heart-searching in the aftermath of Hiroshima.

Rutherford, another farmer's son, regarded Bragg "as a very great man", but Bragg himself remained diffident about the qualities that had raised him to the Presidency of the Royal Society. One evening, opening his study door, his daughter noticed how wearily he looked up from his papers.

"Daddy, need you work so hard?" she cried, and he answered simply, "I must dear; I'm always afraid they'll find out how little I know."

Bragg's last scientific function was a radio discussion with Bernal on living

crystals. It was all about the nature of proteins and viruses. Bragg:

"How do protein molecules get folded? Can no one make a protein?"

Bernal:

"It looks as if all proteins are made by others inside the cells."

Bragg:

"But if one protein must always come from another, how did the first one get there?"

Bernal's answer avoids this paradox.

"One big step towards finding out is getting the structure of proteins and viruses. It's really the problem of the origin of life."

No inkling yet about the role of nucleic acids. Bernal had been Bragg's research student, and Bragg may have had him in mind when he wrote:

"A good research student is like a fire which needs but a match to start it." Quotations such as this one make the book delightful reading.

I was intrigued by the similarities between father and son. They had both read mathematics, but neither was a theoretician at heart, and they solved scientific puzzles in a concrete, visual fashion. The father complained of Planck not bothering to think about

the physical nature of the quantum and to the son quarks had no appeal. To them a structure solved was like an artist's vision, to be described in words of poetic imagery. They felt more at ease with children than adults and preferred to deal with things rather than people. They were no match for politicians: the father was taken for a ride by the higher civil servants when he demanded that science should have a stronger voice in the conduct of the war; the son was helpless among the Byzantine complexities of University politics. The father's faith in God and the goodness of science marks him out as a man of the last century rather than of this one; the son avoided ultimate questions and tended his garden.

What would the reader gain from this book? Much enjoyment; the realisation that success in science can be combined with devotion to human values, and that occasionally the great can also be good and true. □

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DNA inconstancy

Endopolyploidy and Polyteny in Differentiation and Evolution. By W. Nagl. Pp. 283. (Elsevier/North-Holland: Amsterdam, New York and Oxford, 1978.) \$54.50; Dfl.125.

As indicated by its title, this book is concerned with the relationships between two of the central issues in biology—namely, differentiation and evolution. The thread that connects the two, as seen by the author, is the occurrence of DNA variation as an important aspect of both. Evolution has been accompanied by remarkable shifts in the amount, and in the organisation, of basic genomic DNA in different species. Moreover, in very many Orders of animals and plants, there occurs varying degrees and types of ontogenetic elaboration of the basic genome. These are seen in their most obvious expression as polyteny and endopolyploidy, and more subtly, as amplification of certain selected regions of the genome such as the ribosomal cistrons.

To many biologists, such expressions of DNA inconstancy are mere curiosities which, though useful experimentally, serve to emphasise the rule of DNA constancy, with normal mitosis as the orthodox mechanism of DNA growth. Reading this book will do much to redress the balance for, far from being the exception and the

concern of insects and bugs, DNA inconstancy is to be found universally. In fact, after reading this book one feels that the cell nucleus has such a penchant for ontogenetic elaboration that the problem becomes not how or why this occurs, which are matters discussed at length, but how species manage at all to maintain DNA constancy for the purposes of ensuring their continuation.

In all probability it is precisely the failure of species entirely to constrain the exuberance of DNA in this respect that is one of the main driving forces of the evolutionary process. In a very real sense, therefore, the study of ontogenetic DNA variation contains valuable clues to the understanding of phylogenesis and evolution. In other words, speciation and differentiation are two sides of the same coin, which is DNA variation. Along the way to making this point the book commences with a concise and up-to-date account of the organisation of the eukaryotic chromosome and continues with chapters dealing with the occurrence and characterisation of endopolyploid nuclei, the mechanism of endopolyploidisation, further mechanisms of DNA variation, control of endopolyploidisation and polytenisation, functional significance of endocycles, and, finally, evolutionary aspects.

This final chapter brings together the threads running through the previous parts of the book in a far ranging discussion concerned with the meaning of DNA variation in evolution. Com-

mencing with a tabulation of the various mechanisms of, and the terminology for, DNA variation in phylogeny and ontogeny, the author discusses the role of point mutations (adaptation and anagenesis) and of repetitive DNA (speciation and cladogenesis) in the evolutionary process. In particular he emphasises the nucleotypic effects of alterations in DNA and of genomic DNA repatterning as important mechanisms. Nucleotypic effects are considered to be explicable principally in terms of the Britten-Davidson model of gene regulation and the relationship of DNA interspersal patterns to genome size is discussed as a possible nucleotypic effect of DNA mass. Endopolyploidisation and differential DNA replications are considered as evolutionary strategies in light of the fact that, in general, these phenomena achieve their most extreme expression in species with relatively low DNA C values.

The final paragraphs of this chapter set out the author's views concerning the integration of DNA variations during ontogenesis and evolution summarised by what he terms the DNA optimisation model. This illustrates some possible changes in genomic size and organisation during phylogenesis and considers their possible consequences on development. This is altogether a very thought-provoking section of the book which is otherwise an extensive and important review.

Altogether there are 283 pages, of which 49 pages are of references, including the Russian and older German papers, amounting in all to over 1200 references of which 55 are to the work of the author and his colleagues. These are extensively cited and critically discussed and cross-referenced, making this not a book to be read without effort. It is well and profusely illustrated with line diagrams and half-tones, and contains many unique and informative tabulations of data collected from the numerous sources cited. There is a useful subject index and two appendices containing a glossary of terms and further guidance to the methodological literature dealing with the detection of endopolyploidy and differential DNA replication, the preparation and analysis of chromosomes, *in situ* hybridisation, DNA characterisation, and genome organisation analysis.

An indispensable book for the expert but aimed also at a wider readership, it should appeal to students of cytogenetics and molecular biology.

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Receptors and recognition

Receptors and Recognition. Series A. Vol. 5. Edited by P. Cuatrecasas and M. F. Greaves. Pp. 212. (Chapman and Hall: London, 1978.) Hardback £10; paperback £6.

PREVIOUS volumes in this series have contained four or five articles, but this one contains only three, albeit rather longer, contributions. "Relationships in the structure and function of receptors for glycoprotein hormones, bacterial toxins and interferon" by L. D. Kohn is a contribution central to the theme of the series, whereas "Stereoselective recognition in biology" by P. A. Lehmann and "Fluorescence and NMR studies of membranes" by A. G. Lee can be considered to some degree as contributions to the general education of those interested in biological recognition processes.

Lehmann's chapter is a wide-ranging discussion of the ways in which the mutual recognition between macromolecules and small ligands may be influenced by stereochemical variations in the nature of the bound ligand. It draws its examples from an extraordinary diversity of receptor and enzymic recognition systems, thus placing shoulder to shoulder the pheromones of bark beetles, the proteolytic specificity of papain, conformationally hindered thyroxine derivatives and the interaction between avidin and biotin. Not unnaturally, substantial space is given to the author's own 'eudismic' method for deriving quantitative relationships between affinity and stereoselectivity in various series of homologous pairs of stereoisomers.

As there have recently been a considerable number of reviews of the contributions of spectroscopic techniques to understanding of membrane structure, some of them by Lee himself, a chapter on this topic would only seem justified if it emphasised the ways in which such techniques might be helpful to students of biological recognition phenomena. Unfortunately, this is not what is offered. Instead, the chapter opens with good intentions of interpreting NMR data for the uninitiated, but it soon becomes yet another detailed discussion of the spectroscopic characteristics of hydrated lipids, especially dipalmitoylphosphatidylcholine. Proteins, and even receptors, do get briefly discussed in the final section on fluorescence studies, but it seems doubtful whether many general readers will get this far.

Finally, Kohn's contribution takes us to the heart of recent studies on the receptors for a superficially diverse

'family' of ligands (glycoprotein hormones, bacterial toxins and interferon). It is mainly through the work of Kohn and his colleagues, particularly their studies on gangliosides as receptor components and on sequence homology, that these ligands and their receptors can in any sense be regarded as a family. Because of this, the chapter is to be welcomed, but with the slight suspicion that Kohn may, at least sometimes, be adept at seeing unities where none exist. As this is very much the chapter of an enthusiast, it is filled with intriguing snippets of unexplained new information which will keep the

reader's appetite whetted for the next episodes in this intriguing tale.

Overall, therefore, this is an uneven book with too much space devoted to material far from the nitty-gritty of receptors and recognition. Its proof-reading may be a bit better than in earlier volumes (see review in *Nature* 274, 192-193; 1978) but there is still no index (purchasers of volume 6, as yet unpublished, are to get the indices of volumes 1-5 as a bonus). **R. H. Michell**

R. H. Michell is Lecturer in Biochemistry at the University of Birmingham, UK.

Conformational energy surfaces

Biomolecular Information Theory: Studies in Physical and Theoretical Chemistry. By S. Fraga, K. M. S. Saxena and M. Torres. Pp. 271. (Elsevier: Amsterdam, Oxford and New York, 1978.) \$48.75; Dfl.112.

READING this book was a horrible experience. I hasten to add that this was not a fault of the book itself, which has many commendable features. It was merely a manifestation of that recurrent nightmare of textbook writers, a new book with similarities to that which he is in the throws of writing. After some deliberation, it seemed that it would be fun to review a book from an unusually informed viewpoint, but the reader of this review is forewarned that the reviewer may be revealing some of the prejudices of a literary cuckold.

Inevitably, closer inspection revealed many important differences. It is primarily an essay in quantum biochemistry, with emphasis on the conformational energy surfaces of units of protein and nucleic acid molecules. The title is, perhaps, somewhat misleading, as the book does not contain much more about biomolecular information than most biochemical and biophysical textbooks, and there is very little information theory proper. Nonetheless, the first major section provides an illuminating discussion of topics peripheral to an information theory of biological molecules, including the mathematics of the prebiotic origins of self-replicating systems. There is also an entertaining account of the formation of amino acids in the prebiotic soup, and of the occurrence of amino acids in meteorites and Moon dust.

The second major section is largely a vehicle for discussing the stereochemical code of proteins and nucleic acids in terms of the conformational behaviour of low molecular weight analogues of their units. In particular,

N-acetyl N'-methylamide derivatives of amino acids are considered and there is an exhaustive treatment of their conformational energy surfaces as a function of the two major angles of rotation in the backbone. There is also an extensive discussion of the theory and methods (with appendices) for calculating such energy surfaces, and of techniques (such as minimisation and Monte Carlo) for treating more complex energy surfaces. Unfortunately, the authors run the risk of placing too much emphasis on one type of energy calculation, the semi-empirical quantum mechanical PCILO technique of Maigret and Pullman. Indeed, much of the book reads like an encyclopaedia of PCILO results. This gives the text a certain consistency, but the danger of emphasising one type of calculation is that the future usefulness of the book depends to some extent on the validity of that calculation. It was a little one-sided of the authors to opt for PCILO, because the energy surfaces calculated by this method are somewhat anomalous with respect to those derived by the classical studies, as well as by recent empirical crystal-derived potentials and extended basis set *ab initio* calculations. Some CNDO results are reported, although this is one of the few types of calculation which produces similar energy surfaces to PCILO. The consequence of this is that the stereochemical code as viewed by the authors well allows some conformational features not believed by many workers.

Incidentally, the energy maps are presented in the old angle convention, which might cause some confusion in the partially initiated, particularly as the old convention is not used consistently throughout the book.

More generally, the book is pleasant to read, although the jargon is not well suited to biologists. Its mood is technical rather than journalistic ("Valinomycin is a cyclodecapeptide"). A few sections, such as that on "Topological Characteristics of Energy Hypersurfaces", turned out to be more trivial than suggested by a first read.

Some of the mathematical treatments also seemed complicated even to workers in this laboratory who had previously translated equivalent treatments into working computer programmes, although it must be admitted that there are many books which are much worse in this respect. The book seems rightly aimed at theoretical

chemists, intending to introduce them to the biological applications and significance of their work.

In the last analysis, it is a book I would be glad to own.

Barry Robson

Barry Robson is Lecturer in Biochemistry at the University of Manchester, UK.

Electrical properties of biological cells

Impedance Measurements in Biological Cells. By O. F. Schanne and E. R. P. Ceretti. Pp. 430. (Wiley-Interscience: New York and Chichester, UK, 1978.) £21.20.

THERE exist two major schools of thought about the measurement of the electrical properties of biological cells. One owes its origin to a physicomathematical approach using the concept of impedance. Typically, this involves investigating the response of the cell to a wide range of frequencies followed by an attempt to model the response physically with an electrical equivalent circuit. The second school has typically been more interested in measuring the membrane resistance and in relating this to the physicochemical mechanisms determining the membrane permeability to ions. This has usually involved measuring the response to step changes of potential or current. Of course, the two approaches are complementary but it is nevertheless true to say that in the past the two schools have sometimes been far apart; even when they have come together on the same problem the interpretations have retained characteristic differences. I myself think that this is exemplified by the major difference between the approaches of Cole and of Hodgkin and Huxley to the analysis of the squid nerve fibre. The same properties of the cell lead Cole to represent the cell as behaving like an inductance, whereas Hodgkin and Huxley represented the inductance by a time dependent permeability change. A trivial difference in one sense, as the representations are equivalent. A major difference from an heuristic viewpoint since one's choice of representation does determine what one thinks is worth doing in future experimentation.

The authors of this book are well aware of this difference of approach; indeed they refer to the need to "show that the results obtained by the two groups complement each other". The book begins with a valuable treatment of the experimental and theoretical methods employed. This is followed by sections on impedance measurements in nerves, in muscles, in epithelia and on

cell suspensions. In the process, a vast amount of an even vaster literature is reviewed. My major criticism of the book is that the attempt to show that the results obtained by the two groups complement each other is not very well sustained. Many parts of the later chapters are simply reviews written largely in terms of the outlook of the original authors. My second criticism is that this review of the literature does not extend much beyond 1972 in many chapters.

Anyone who has written a book of

Techniques of centrifugation

Centrifugal Separations in Molecular and Cell Biology. Edited by G. D. Birnie and D. Rickwood. Pp. 327. (Butterworths: London and Boston, 1978.) £14; \$28.

THIS book is concerned mainly with work that is done using preparative rotors. It proceeds from the theoretical background to consider in turn rate-zonal centrifugation, separations in zonal rotors, and isopycnic centrifugation.

The main chapter on theory acknowledges the antipathy of some biologists towards mathematics by relegating some sections to an appendix. In other chapters considerable use is made of simplified equations to illustrate principles, and even those who have not followed the derivations are encouraged to avail themselves of the resultant expressions much as they might do the time shown on an electronic watch. The design of their experiments, often an art as much as a science, may thereby be improved. Those practitioners who feel, for financial or other reasons, that their machines ought to have a shorter working week and higher payload, may benefit substantially from such considerations. Theory properly applied enables extra information, such as sedimentation coefficients, to be obtained from preparative experiments, but the authors wisely include a caution about the importance and sometimes difficulty of maintaining adequate temperature control.

this size and extent will readily understand the problems but, whether these are primarily those of the authors or publishers, the reader should be warned that he cannot expect the treatment of certain areas to be very up-to-date. There are many examples of this. Thus, in the section on passive properties of cardiac muscle, the important work of Mobley and Page in 1972 is included, but the equally important contribution of Hellam and Studt in 1974 is omitted. In the section on skeletal muscle, Costantin's work up to 1970 is included but not his 1973 work with Taylor, nor his valuable 1975 review. I cannot therefore imagine that many private buyers will find the book sufficiently useful to justify buying it, but as a library volume for reference purposes, it may still be worth considering.

Denis Noble

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The chapter on isopycnic systems emphasises the advantages of fixed angle rotors. Although it mentions lipoproteins specifically, it fails to draw attention to the satisfactory separations of glycoproteins that have been achieved with this technique. Ancillary equipment receives some attention, but the survey of types of gradient-forming devices does not mention the versatile apparatus of Hegenauer *et al.* The percipient reader will notice a few blemishes, but these are rarely misleading. In chapter 2, η is used in the conventional way for viscosity, but in later chapters symbolises refractive index. Equations 6.7 and 6.8 were first derived by Ifft *et al.* in 1961, not 1970. In chapter 8 in several places the "e" is omitted from "Weiss". The most intriguing observation is "esoteric" on p189, a term that invites adoption by anyone who can muster a credible claim. Though the chapter on analytical ultracentrifugation is strictly within the scope of the title of the book, readers interested in these matters will probably seek a more specialised text. Where repetition occurs, its objective seems to be to make each chapter fairly self-contained.

This volume is well-written, is full of practical tips, and has concentrated from diverse sources a collection of tables on the characteristics of many types of biological particles, macromolecules, gradient materials, rotors and tubes. It will afford something of value to even the most experienced worker.

P. A. Charlwood

P. A. Charlwood is on the scientific staff of the MRC at the National Institute for Medical Research, London, UK.

Nutritional problems of the Third World

Development, Reform, and Malnutrition in Chile. By P. Hakim and G. Solimano. Pp. 91. (MIT Press: Cambridge, Massachusetts, and London, 1978.) \$10; £7.

THERE is a real need for examples of national programmes that actually work within the economic restraints of developing countries. Development should benefit people, and the best measure of an improved quality of life is the elimination of malnutrition, which results primarily from poverty and ignorance. It is not enough to increase food production when people cannot buy it, nor to raise incomes when people do not know what to buy. The Chilean example has much to commend it, as an attempt was made to reduce malnutrition at a time of social change. The Allende régime lasted three short years, but during this period it attempted fundamental changes in economic policy by bringing large sections of industry under state ownership and extending land reform. A general redistribution of income was achieved, unemployment was reduced, and the social services were made more available to the low income groups. Food consumption increased by about 10%, although largely as a result of food imports, and the distribution of free milk by the Government was trebled. In other words all the elements for the successful improvement of nutritional status appeared to be present. The question is, did it happen?

The authors of this book are frankly pessimistic, although a proper evaluation was not carried out. The free milk scheme, the major topic of their book, provided distribution from schools and clinics which were not available to all the population thereby giving preference to the richer urban community: the milk reached only 70% of the eligible beneficiaries. Although it was provided to the most vulnerable age groups, it was often shared within the family and occasionally sold. Nevertheless, 40,000 tonnes of dried milk was distributed annually. A careful analysis of Chile's nutritional problems reveals, however, that they are due to a low consumption of food rather than a lack of particular nutrients and the emphasis on protein should be challenged.

It is self-evident that the primary need of starving children is food. In retrospect, the authors recognise the lack of scientific rationale for the milk programme and admit that the present oppressive government, which is now

distributing 25,000 tonnes but with added fat, might be better advised. The promotion of protein-rich foods derives from old-fashioned nutritional ideas which were outmoded at the time of Allende, and that of free milk from socio-political targets which were unrealistic for the economy. All of this is examined in detail in the book.

It could be argued that the period was too short to expect any change in nutritional status, but this is not so. The growth rate of deprived children shows a marked improvement when they are well fed: a nutritional programme in Korea has led over seven years to a national increase of 3 cm in average height and 1 kg in average weight of children. Also, in Ethiopia an applied nutritional programme halved the infant mortality rate in three years. Simple statistics of this kind are essential for evaluation—relatively easy to obtain, but not apparently available from Chile over the relevant period. Such data may not be as easy to interpret as the results of laboratory experiments where everything is controlled, but it is more satisfying to try to explain successes than failures.

The authors are well aware of the economic, educational and medical

factors influencing nutritional status and suggest that all were improving during Allende's time but the reader, may not be entirely convinced. In particular, although they distinguish between preventative and curative medicine, they do not stress the difference between the strictly medical and public health engineering, for example, water and sanitation. The latter is probably more effective than vaccination programmes albeit much more expensive. Similarly the educational programmes were largely through the mass media and possibly did not reach or convince the most socially deprived. Finally, action to redistribute wealth which satisfied the intellectuals, may not have been as effective as claimed. But certainly the position has deteriorated since 1973 and the brave efforts of Allende reported here are worthy of this objective documentation.

The book will interest those concerned with recent Chilean history, but will be most valuable to those grappling with nutritional problems of the Third World.

D. S. Miller

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Chemical and enzymic catalysis

Transition States of Biochemical Processes. Edited by R. D. Gandour and R. L. Schowen. Pp. 616. (Plenum: New York and London, 1978.)

THE theory of absolute reaction rates, commonly known as transition state theory has been perhaps the most influential principle in the development of our knowledge of reaction mechanisms in solution. This book consists of a series of chapters written by 21 authors on the theories and methods used to investigate chemical and enzymic catalysis.

The book is written in four parts. In part I the assumptions in transition state theory are examined and its application to chemical kinetics and mechanisms discussed. When enzyme-catalysed reactions are considered, the fundamentalist view is adopted, namely that catalysis originates from stabilisation of the transition state through binding to the catalyst.

In part II, which is the longest section of the volume, the main techniques used to investigate transition state structure and properties are introduced. Rightly this section is dominated by the application of isotope effects. Primary, secondary and solvent isotope effects of hydrogen are dis-

cussed as well as the heavy-atom isotope effects on carbon, nitrogen, oxygen and chlorine. The first chapter of this section, however, introduces the quantum mechanical procedures which have been used to investigate enzymic transition states and reaction pathways. The last two chapters review the application of magnetic resonance techniques to investigate enzyme-ligand complexes and the mapping of reaction pathways from X-ray crystallographic data.

In part III, well studied reactions are discussed which are biochemically important. These include acyl, methyl, and phosphoryl transfer, acetal hydrolysis, decarboxylations and relevant models of enzymic reactions. In the final part, the use of inhibitors as tools to elucidate the sources of enzymic catalysis are discussed and the application of transition state information to the design of potent drugs is described.

Thus the book ranges over all the major disciplines which have contributed to the investigation of chemical and enzymic mechanisms. The editors have selected an excellent array of authors and have welded their contributions together into a text which will be of value to a wide range of chemists and biochemists.

G. Lowe

G. Lowe is Lecturer in Organic Chemistry at the University of Oxford, UK.

announcements

Awards

Guido Brunner, Commissioner of the European Community responsible for energy policy, has been awarded this year's Melchett Medal of the Institute of Fuel. The award is made annually "for outstanding work, whether in research, administration, construction, or other professional activity involving the scientific preparation or use of fuel, the results of which have recently been made available for the benefit of the community".

Dr David Arthur Davies, Secretary-General, World Meteorological Organization, has been awarded the Gold Medal of Merit of the Czechoslovak Academy of Sciences, Prague.

The 1977 Kalinga Prize has been awarded by a UNESCO international jury to **Fernand Seguin** in recognition of his life's work in popularising science.

Nominations are invited for the 5th Marconi International Fellowship Award. The Fellowship aims to commission creative scientific works that will add to knowledge and understanding of how communications sciences and technologies can be applied to improvement of human life. Contact Walter Orr Roberts, 1919 Fourteenth St, No. 811, Boulder, Colorado 80302.

The Council of the Royal Society are inviting nominations for the 1979 Royal Society Esso Award for the Conservation of Energy which should be sent to R. W. J. Keay, The Royal Society, 6 Carlton House Terrace, London SW1, UK, by 6 February 1979. The award is for outstanding contributions to the advance of science or engineering or technology leading to the more efficient conversion or use of any form of energy, and consists of a gold medal and a prize of £1,000.

The World Phosphate Rock Institute has created the Imphos Award to extend the impact of the First International Congress on Phosphorus Compounds and to foster research on their non-fertiliser uses. The first award will be granted in 1979. Applications to IMPHOS, 8 rue de Penthièvre, 75008 Paris, France.

The Foulkes Foundation is a registered charity which aims to further medical research. The fellowship scheme provides financial support for recently qualified science graduates with research experience who want to study medicine and for medical graduates who want to take a science degree. In both cases the graduates must intend to do medical research after qualification. For further information contact: The Secretary, Foulkes Foundation Fellowship Scheme, The Ciba Foundation, 41 Portland Place, London W1, UK, enclosing a s.a.e. The closing date for applications is 31 March 1979. The 1978 Foulkes Foundation Fellows are **G. Belcher, C. J. Derrett, Catherine T. Elder, T. S. J. Elliott, P. R. Hearn, M. J. C. Lemon, Isabella McMillen and D. C. Talbot**.

Meetings

18–20 December, **Palaentological Association Annual Meeting**, Keele (Dr P. D. Lane, Dept of Geology, University of Keele, Staffs, UK).

4–5 January, **Laser-Plasma Interactions**, Bangor (T. P. Hughes, Dept of Physics, University of Essex, Colchester, Essex, UK).

23–25 January, **Inter-Agency Group on Biometeorology**, Geneva (WMO, Case Postale No. 5, CH-1211, Geneva 20, Switzerland).

29 January–2 February, **RA IV Working Group on Hydrology**, Mexico City (WMO, Case Postale, No. 5, CH-1211, Geneva, Switzerland).

29 January–24 February, **Approaches to the Molecular Genetics of Oncogenic Viruses by DNA Cloning and DNA Injection into Mice Blastocysts**, Rome (Prof Enrico Calf, Centro Acidi Nucleici del CNR, Via G. Romagnosi 18/A, 00196 Rome, Italy).

12 February, **Second-order Logic and the Foundations of Mathematics**, London (The British Society for the Philosophy of Science, Mr A. Young, School of Social Sciences, University of Sussex, Falmer, Brighton, UK).

15–16 February, **Immunodeficient Animals in Cancer Research**, London

(The Information Officer, MRC laboratory Animals Centre, Woodmansterne Road, Carshalton, Surrey, UK).

12–23 February, **World Climate Conference**, Geneva (WMO, Case Postale No. 5, CH-1211, Geneva 20, Switzerland).

12 March, **AGM The British Society for the Philosophy of Science**, London (Mr A. Young, School of Social Sciences, University of Sussex, Falmer, Brighton, Sussex, UK).

20 March, **Mathematical Modelling of Large Scale Accidents and the Environment**, Cambridge (The Secretary, The Institute of Mathematics and its Applications, Maitland House, Warrior Square, Southend-on-Sea, Essex, UK).

21 March, **Models of Estuaries: Flow and Pollution**, Edinburgh (The Secretary, The Institute of Mathematics and its Applications, Maitland House, Warrior Square, Southend-on-Sea, Essex, UK).

26–30 March, **Electron Spin Resonance of Transition Metal Ions in Inorganic and Biological Systems**, Nottingham (Miss G. B. Howlett, The Chemical Society, Burlington House, Piccadilly, London W1, UK).

3–5 April, **The Chemical Society and the RIC, Annual Chemical Congress**, Bristol (Dr John Gibson, The Chemical Society, Burlington House, Piccadilly, London W1, UK).

9–11 April, **11th National Atomic and Molecular Physics Conference**, Liverpool (The Institute of Physics, 47 Belgrave Square, London SW1, UK).

27–31 May, **International Prostaglandin Congress**, Washington, D.C. (Prof J. Martyn Bailey, PO Box 40591, Georgetown University Medical Center, Washington, D.C. 20016).

29 May–1 June, **Radioimmunoassay of Drugs and Hormones**, Gardone Riviera (Fondazione Giovanni Lorenzini, Via Monte Napoleone, 23 20121 Milan, Italy).

8–10 June, **Multidisciplinary Aspects of Brain Tumour Therapy**, Pavia (Fondazione Giovanni Lorenzini, Via Monte Napoleone, 23, 20121 Milan, Italy).

11–15 June, **14th International Microwave Power Symposium**, Monaco (Comité Français d'Electrothermie, 79 rue de Miromesnil, 75008 Paris, France).

11–15 June, **3rd International Symposium on Purine Metabolism in Man**, Madrid (Unidad Metabolica, Fundacion Jimenez Diaz, Universidad Autonoma, Avida, Reyes Catolicos, 2, Madrid-3, Spain).

26–28 June, **Power from Sea Waves**, Edinburgh (The Secretary, The Institute of Mathematics and its Applications, Maitland House, Warrior Square, Southend-on-Sea, Essex, UK).

18–20 July, **Internal Friction and Ultrasonic Attenuation in Solids**, Manchester (The Institute of Physics, 47 Belgrave Square, London SW1, UK).

15–19 July, **International Conference on Clinical Aspects of Cyclic Nucleotides**, Vail, Colorado (Howard Sands, National Jewish Hospital and Research Center, 3800 East Colfax Ave, Denver, Colorado 80206).

16–20 July, **Molecular Biology of Small DNA Tumour Viruses** (This replaces the Cold Spring Harbor DNA Tumour Virus Meeting) Cambridge (ICRF DNA Tumour Virus Meeting Secretary, ICRF, Lincoln's Inn Fields, London WC2, UK).

23–26 July, **Synthesis in Organic Chemistry**, Cambridge (Dr Malcolm Robinson, The Chemical Society, Burlington House, Piccadilly, London W1, UK).

20–23 August, **1st European Symposium on Organic Chemistry**, Cologne (Dr W. Fritzsche, c/o Gesellschaft Deutscher Chemiker, PO Box 90 04 40, D-6000 Frankfurt/Main 90, FRG).

3–7 September, **Practical Applications and Design of Fibre Composites**, Cambridge (Dr Peter Beaumont, Dept of Engineering, University of Cambridge, Trumpington St, Cambridge, UK).

22–25 October, **Conference on Reclamation of Contaminated land**, Eastbourne (Miss J. Bovier, Conference Secretary, Society of Chemical Industry, 14 Belgrave Square, London SW1, UK).

10–16 February 1980, **6th International Congress of Endocrinology**, Box 611E, GPO, Melbourne 3001, Australia.

7–17 July 1980, **26th International Geological Congress**, Paris (Secretariat Général du 26ème Congrès géologique international, Maison de la Géologie, 77–79, rue Claude Bernard, 75005 Paris, France).

22–27 July 1980, **International Cyclic Nucleotide Conference**, Brussels (Dr J. E. Dumont, IRIBHN, School of Medicine, 2 rue Evers, B-1000 Brussels, Belgium).

3–9 August 1980, **16th International Congress of Entomology**, Kyoto (16th International Congress of Entomology, c/o Kyoto International Conference Hall, Takara-ike, Sakyo-ku, Kyoto 606, Japan).

17–22 August 1980, **6th International Histochemistry and Cytochemistry Congress**, Brighton (The Administrator, Royal Microscopical Society, 37/38 St Clements, Oxford, UK).

22–26 September 1980, **International Union for Vacuum Science, Technique and Applications**, Cannes (Société Française du Vide, 19 rue du Renard, 75004 Paris, France).

22–26 September 1980, **3rd International Congress on the History of Oceanography**, Woods Hole (Daniel Merriman, 298 Sperry Road, Bethany, Connecticut 06525).

21–28 August 1981, **13th International Botanical Congress**, Sydney (Dr W. J. Cram, University of Sydney, New South Wales 2006, Australia).

Reports & publications

UK & Ireland–October

Pharmaceutical Prices: A Continental View. By Dr. Klaus von Grebner. Pp. 24. (Reprinted in translation from an article in *Die Pharmazeutische Industrie*.) (London: Office of Health Economics, 162 Regent Street, W1, 1978.) £1. [2010]

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Information Privacy: Integrity, Availability, Security. Vol. 1, No. 1, September 1978. Edited by D. Sizer. Pp. 1-48. Published every other month. Annual subscription: £35; \$91. (Haywards Heath, Sussex: IPC Business Press (Sales and Distribution), Ltd., Oakfield House, Perrymount Road, 1978.) [2310]

Tropical Products Institute. The Small-Scale Manufacture of Soap: an Economic Study. By Penelope A. Mars. (G118). Pp.v+46. (London: Tropical Products Institute, 56/62 Gray's Inn Road, WC1, 1978.) £1. [2310]

Institute of Geological Sciences. Annual Report for 1977. Pp.x+226. (London: Institute of Geological Sciences, 1978.) £4.50. [2410]

Agricultural Development and the Rural Poor: Declaration of Policy and Guidelines for Action. Edited by Guy Hunter. Pp.xii+113. (London: Overseas Development Institute, 10-11 Percy Street, W1, 1978.) £1.50. [2510]

Thames Water—Annual Report and Accounts for the year ended 31st March 1978. Pp. 76. (London: Thames Water Authority, New River Head, Rosebery Avenue, EC1, 1978.) [2510]

Council for National Academic Awards. Directory of Postgraduate and Post-Experience Courses, 1978-79. Pp. 88. Directory of First Degree Courses, 1978-79. Pp. 184. (London: Council for National Academic Awards, 344-354 Gray's Inn Road, WC1, 1978.) [2510]

The Polytechnic of North London. Prospectus 1979-80. Pp. 114. (London: The Polytechnic of North London, Holloway, N7, 1978.) [2510]

Identification of Larval Ticks Found on Small Mammals in Britain. By K. R. Snow. Pp. 14. (Bury St. Edmunds, Suffolk: Publications Officers, Mammal Society, Larkmead, Barton Mills, 1978.) 25p+10p. P+p. [2710]

Natural Environment Research Council. Chemistry in the Institute of Terrestrial Ecology. By S. E. Allen. Pp. 31. (Cambridge: Institute of Terrestrial Ecology, 68 Hills Road, 1978.) 75p net. [3010]

National Coal Board. Mining Research and Development Establishment. Projects 1977/78. Pp. 49. Annual Report, 1977/78. Pp. 28. (Burton-on-Trent, Staffs.: Mining Research and Development Establishment, Ashley Road, Stanhope Bretby, 1978.) [3110]

Other countries–October

Application of the International Classification of Diseases to Dentistry and Stomatology, ICD-DA. Second edition. Pp. 144. (Geneva: WHO; London: HMSO, 1978.) Sw.fr.20; \$11. [2010]

Smithsonian Contributions to Zoology, No. 278: A Catalogue of the Type-Specimens of Recent Cephalopoda in the National Museum of Natural History. By Clyde E. Roper and Michael J. Sweeney. Pp.iii+19. (Washington, DC: Smithsonian Institution Press, 1978. For sale by US Government Printing Office.) [2310]

The European Community Today and Tomorrow. Pp. 64. (Luxembourg: Office for Official Publications of the European Communities, Boite Postale 1003, 1978.) Bfr.32; 60p. [2310]

United States Department of the Interior: Geological Survey. Professional Paper 935-C: Crystallization and Differentiation of the Alae Magma, Alae Lava Lake, Hawaii. By Thomas L. Wright and Dallas L. Peck. Pp.iv+20. Professional Paper 1053: Potential Effects of Deep-Well Waste Disposal in Western New York. By Roger M. Waller, John T. Turk and Robert J. Dingman. Pp.v+39. Professional Paper 1055: Reconnaissance Geology and Geochronology of the Precambrian of the Granite Mountains, Wyoming. By Zell E. Peterman and R. A. Hildreth. Pp.iii+22. (Washington, DC: US Government Printing Office, 1978.) [2310]

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The Worldwide Loss of Cropland. By Lester R. Brown. Pp. 48. (Worldwatch Paper No. 24.) (Washington, DC: Worldwatch Institute, 1776 Massachusetts Avenue, N.W., 1978.) \$2. [3110]

Smithsonian Contributions to Zoology, No. 251: A Revision of the North American Moths of the Superfamily Eriocraniioidea with the Proposal of a New Family, Acanthopteroctetidae (Lepidoptera). By Donald R. Davis. Pp.ii+131. (Washington, DC: Smithsonian Institution Press, 1978. For sale by US Government Printing Office.) [3110]

Recent scientific and technical books

Applied biology

- ANDERSEN, K. Lange, MASIRONI, R., RUTENFRANZ, and SELIGER, C. In collaboration with DEGRE, S., TRYGG, K., and ORGIM, M. *Habitual Physical Activity and Health*. (WHO Regional Publications, European Series No. 6.) Pp.188. ISBN-92-9020-106-1. (Copenhagen: WHO, Regional Office for Europe; London: HMSO, 1978.) Sw.fr.25; \$12.50.
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